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**Molecular Characterization of *XvPer1*, a
novel antioxidant enzyme from the
resurrection plant *Xerophyta viscosa*,
and *AC3*, a LEA-like protein from
*Arabidopsis thaliana***

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**To Rashaad,
Thank you for your love and companionship**

Finally, I dedicate this work to my parents. You are far in distance, but closest to my heart. Thank you.

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ABBREVIATIONS

ABA	Absciscic acid
ABRE	ABA response element
APX	Ascorbate peroxidase
BSA	Bovine serum albumin
Ca(NO ₃) ₂	Calcium nitrate
cDNA	Copy DNA
cm	Centimeter
CoCl ₂	Copper chloride
CTAB	Hexadecyltrimethylammoniumbromide
CuSO ₄	Copper sulphate
Cys	Cysteine
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
DRE	Dehydration response element
DTT	Dithiothretol
EDTA	Ethylenediaminetetra-acetic acid
ELISA	Enzyme-Linked immunosorbent assay
EtBr	Ethidium bromide
g	Grams
gfp	Green fluorescent protein
GS	Goat serum
H3	Histone calf thymus type III
H ₃ BO ₄	Boric acid
HCL	Hydrochloric acid
HM	Homozygous
H ₂ O ₂	Hydrogen peroxide
IPTG	isopropyl-β-D-thiogalactoside
Kb	Kilobase
KCl	Potassium chloride
kDa	Kilodalton
KNO ₃	Potassium nitrate

KOH	Potassium hydroxide
L	Liter
LB	Luria broth
LEA	Late embryogenesis abundant
M	Molar
MCS	Multiple cloning site
MFO	Mixed-function oxidation
mg	Milligram
µg	Microgram
MgSO ₄	Magnesium sulphate
ml	milliliter
mM	Millimolar
µM	Micromolar
MS	Murashige and Skoog media
NaH ₂ PO ₄	Sodium hydrogen phosphate
Na ₂ MoO ₄	Sodium molybdate
NaOH	Sodium hydroxide
NaCl	Sodium chloride
Ni-NTA	Nickel-nitriloacetic acid
NLS	Nuclear localization signal
O/N	Overnight
ORF	Open reading frame
PB	Phosphate buffer
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PMSF	Phenyl methyl sulfonyl fluoride
PNS	Plant nutrient agar medium
POPP	Protein or Oligonucleotide Probability Profile
Prx	Peroxiredoxin
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolutions per minute
RT	Room temperature
RT-PCR	Reverse transcription-polymerase chain reaction

RWC	Relative water content
SDS	Sodium deodecyl sulphate
SDS-PAGE	Sodium Deodecyl Sulphate Polyacrylamide Gel Electrophoresis
SD-U	Minimal media without uracil
SWC	Soil water content
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
tBOOH	ter-butylhydroperoxide
UV	Ultra violet
v/v	Volume per volume
WT	Wild type
w/v	Weight per volume
ZnSO ₄	Zinc sulphate

ABSTRACT

By differential screening of a cDNA library of the resurrection plant *Xerophyta viscosa*, a cDNA termed *XvPer1* was isolated for its over-expression under dehydration stress. Analysis of the cDNA sequence indicated a hydrophilic protein of a predicted Mw of 24.2 kDa with high identity to plant 1-Cys peroxiredoxins, a novel antioxidant enzyme. Southern blot analysis revealed that *XvPer1* was most probably a single copy gene. The polypeptide sequence had significant identities (~70%) with other recently identified plant 1-Cys peroxiredoxins with an absolutely conserved active site (PVCTTE). The protein sequence also had a putative bipartite nuclear localization signal.

Northern blot analysis revealed that *XvPer1* steady-state mRNA transcript was absent in fully hydrated *X. viscosa* leaf tissue but levels increased in tissues subjected to dehydration. Its expression was also induced by heat stress (42°C), high light intensity (1500 $\mu\text{mol m}^{-2}\text{s}^{-1}$), and when treated with abscisic acid (100 μM ABA) and sodium chloride (100 mM). Western blot analyses of the *XvPer1* protein correlated with the patterns of expression of *XvPer1* transcripts under the different stress conditions except for high light stress.

Immunolocalization studies using immunofluorescence on dehydrated *X. viscosa* leaf tissue and chromogenic immunodetection on ABA treated leaf tissue both localized *XvPer1* mainly to the nucleus and to some areas of the cytoplasm.

Transgenic *Zea mays* plants constitutively over-expressing *XvPer1* were generated. Analysis of the maize transgenics showed no improvement under drought stress but transgenic plants showed a growth advantage over control plants when subjected to high light stress (1000 $\mu\text{mol m}^{-2}\text{s}^{-1}$). *XvPer1* is the first 1-Cys peroxiredoxin found to be expressed in vegetative tissues in angiosperms. All others have been shown to be exclusively seed-specific. *XvPer1* is therefore thought to protect cellular structures against damage caused by the generation of reactive oxygen species (ROS) possibly by protecting DNA against nicking. More transgenic studies are underway to further characterize this antioxidant enzyme.

A late embryogenesis abundant (LEA)-like protein from *Arabidopsis* was isolated for its ability to confer H₂O₂ tolerance to a yeast mutant susceptible to oxidative stress. Analysis of its cDNA sequence revealed that it belonged to group 3 LEAs out of 6 groups of LEA proteins identified so far. The deduced amino acid sequence coded for a small (10.3 kDa) hydrophilic protein and showed ~60% identity to other group 3 LEAs. Northern blot analysis showed that unlike most LEAs, *AC3* was not expressed in *Arabidopsis* seeds under controlled conditions but was expressed in roots and flowers. The *AC3* steady-state mRNA level was found to increase in response to dehydration. The kinetic of the induction during dehydration corresponded with drought inhibition of photosynthesis. *AC3* expression was also induced by the stress hormone ABA (100 μ M) and by exogenous application of the pro-oxidants diamide (1 mM), H₂O₂ (10 mM), menadione (50 μ M) and paraquat (100 μ M). Under dehydration stress, the *AC3* steady-state mRNA level was highly induced in the ABA insensitive mutant *abi1-1* and the ABA deficient mutant *aba1-1*.

Functional studies by yeast complementation of an oxidative stress sensitive mutant *Δyap1* showed that the expression of the *AC3* protein increased tolerance to oxidative stress damage caused by diamide, menadione and ter-butylhydroperoxide. Polymerase chain reaction was used to identify homozygous lines of an *AC3* knock-out *Arabidopsis*. Under conditions of dehydration stress, *AC3* steady-state mRNA level was increased in the WT but not in the knock-out plants. Under hydrated conditions, the HM *AC3* knock-out plants had better photosynthetic efficiencies than WT plants. However, this was reversed as the plants dehydrated. No difference in total protein and chlorophyll contents were detected between the WT and knock-out plants. *AC3* induction was found to increase under dehydration stress in *Arabidopsis* ABA-insensitive and ABA-deficient mutants. Transgenic *Arabidopsis* plants over-expressing *AC3* and antisense *AC3* plants have been generated for further functional analyses.

We postulate that *AC3* perform a protective function of proteins, possibly proteins involved in photosynthesis, in roots and shoots under normal conditions. In certain stress conditions, shoot-to-root signalling induces *AC3* expression by both an ABA-dependent but mainly by an ABA-independent pathway in leaves and protects the

latter from oxidative damage caused by the accumulation of superoxides. Transgenic studies are underway to further characterize *AC3*.

The Arabidopsis *AtPer1* cDNA coding for a 1-Cys peroxiredoxin and its orthologue *XvPer1* from the resurrection plant *X. viscosa*, share 72% identity at the amino acid level. *AtPer1* was isolated by RT-PCR on cDNA generated from mRNA isolated from Arabidopsis seeds. Sequence analysis revealed that it is 648 bp long and codes for a protein of 216 amino acids with a molecular weight of 24 kDa.

Both RT-PCR and northern blot analysis confirmed that *AtPer1* steady-state mRNA transcript was present only in Arabidopsis seeds under optimal growth conditions. Unlike *XvPer1*, *AtPer1* was not induced by dehydration stress.

Transgenic Arabidopsis plants over-expressing the *X. viscosa* orthologue, as well as Arabidopsis plants under-expressing its own 1-Cys peroxiredoxin were developed. T₁ generation seeds for both transgenic types have been collected for further analysis.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

As we enter the 21st century, the prospects for feeding the ever increasing world population are being portrayed as a daunting task (Dyson, 1999). For example, the world's population is growing faster than cereal production and has been doing so since the early 1980s. The rate of growth of world cereal yields is also said to be declining. There is a very strong implication that this decline is due to increasing environmental production constraints. Figure 1.1 shows the world per-capita cereal production from 1951-1997.

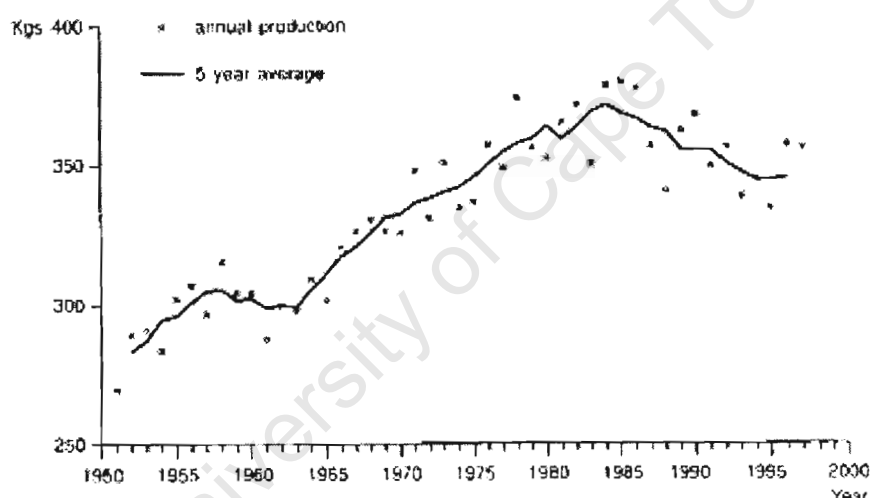


Figure 1.1. World per-capita cereal production, 1951-1997. Averages for 1952 and 1996 are calculated from data for 1951–1953 and 1995–1997, respectively. Here and subsequently cereal data are expressed in production terms (Dyson, 1999).

If regional trends are considered, sub-Saharan Africa has done very poorly in terms of food production (Fig. 1.2). The explanation for this includes political instability, neglect of agriculture by governments, environmental constraints, and despite its many health problems (including the AIDS epidemic), an extremely rapid population growth. Sub-Saharan Africa has also missed out on most 'Green Revolution' technical developments that have boosted agricultural production elsewhere.

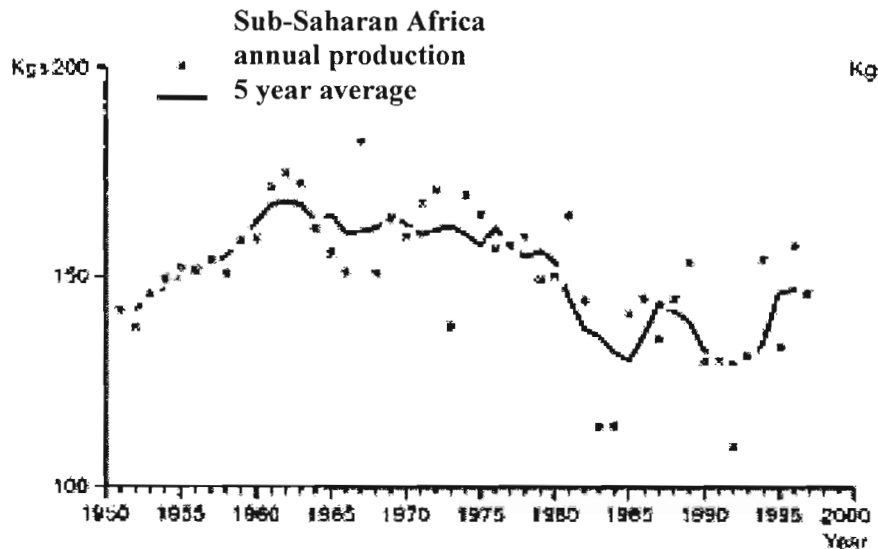


Figure 1.2. Per-capita cereal of the sub-Sahara African region, 1951-1997. Averages for 1952 and 1996 are calculated from data for 1951–1953 and 1995–1997, respectively (Dyson, 1999).

The challenge over the next 50 years will not only be to feed more people, but to also take into account the following:

- There will be less arable land. Overplowing, overgrazing and deforestation have caused soil erosion to exceed soil formation. Africa is particularly hard hit, where soil is shallow to begin with (Kishore and Shewmaker, 1999).
- There will be fewer resources, particularly non-renewable resources such as phosphorus and potassium, which go into fertilisers.
- There will be less water, and the quality of the water available will be reduced as demand increases.

An array of new and improved technology is needed to help address these challenges. Fortunately, we are now in an epoch rich with opportunity for breakthrough technologies, termed the information revolution (Kishore and Shewmaker, 1999). Information-driven agriculture has two components. The first is information based on genomics, the study of genes. The second is based on the silicon revolution. Computers and modern electronic systems are being used for precision agriculture to maximise outputs.

Biotechnology has also developed rapidly in the last two decades. It is a technology that has greatly helped our fundamental ability to precisely introduce genetic changes into an organism. Plant biotechnology in particular has evolved rapidly in the last 15 years. It can substantially improve plant breeding, which was based on our ability to harness genes into plants either by sexual crossing or laboratory techniques such as cell fusion.

Biotechnology can be used to improve agronomic traits. Since 1995, major products with improved agronomic traits have been introduced in the USA and other parts of the world. They were mostly single traits where the introduction of a single gene has had a dramatic positive impact on grower productivity. This has led to widespread acceptance and use of genetically improved crops in the United States. One example is the YieldGuard corn, which uses a plant-modified version of the gene encoding an insecticidal protein from a naturally occurring bacterium, *Bacillus thuringiensis*, to help the plant resist the European corn borer (Estruch et al., 1997). The YieldGuard gene significantly reduces the damage caused by the corn borer. Several other products have been introduced into the market-place including Roundup Ready canola, cotton and corn, New Leaf potato, virus-resistant squashes and melons.

Biotechnology can also be used to improve grain quality or output traits (Kishore and Shewmaker, 1999). Where grains are used for animal feed, they can be engineered for high oil and amino acid contents. Plants can also be developed to use as factories. They are nature's best manufacturing system. An important example is high carotenoid canola, rich in β -carotene, a precursor to vitamin A. Most western countries address the problem of vitamin A needs of humans by fortifying milk. However, this is an impractical system in most parts of the world. Essentially populations of all countries of Latin America, Asia and Africa are clinically or sub-clinically deficient in vitamin A (Underwood and Arthur, 1996). Fruits and vegetables with high carotene contents are not readily available to poor people. One of the most important contributions that biotechnology can make to world health is to produce crops naturally fortified with this essential nutrient.

Facing the increasing global fresh water scarcity, many scientists around the world are using biotechnology to engineer transgenic drought resistant plants. Salt tolerance is also an important trait due to high salt levels in large areas of arable land. However, the development of transgenic stress tolerant plants is very slow, which is probably due to the lack of knowledge about the physiological and biochemical mechanisms of stress tolerance in plants. Therefore a thorough study of the mechanisms of stress tolerance in plants, especially those plants which have specialised in stress tolerance, is required. Such plants which have adapted themselves to survive in stressful environments, like the resurrection plants, are good sources of genetic material for these studies.

1.1 PLANT RESPONSES TO OSMOTIC STRESS

As sessile organisms, plants have developed adaptive strategies to cope with environmental stresses. Drought tolerance in plants denotes their ability to survive periodic or episodic lack of moisture in the environment (Balsamo et al., 2005). Most plant species fall into the category of drought tolerators. On the other hand, desiccation tolerance, i.e., the ability to survive drying to below 20% relative water content (RWC), is rare in seed plants but more common among non-vascular plants and mosses. Most plants respond to loss of water by osmotic adjustment. This is an acclimation where the plant lowers its osmotic potential in response to a drought and in this way maintains turgor despite a lower water potential. Under osmotic stress, plants exhibit a wide range of responses at the molecular, cellular, and whole plant levels (Hasegawa et al., 2000). Cellular water deficit results in a concentration of solutes, changes in cell volume and membrane shape, disruption of water potential gradients, loss of turgor, disruption of membrane integrity, and denaturation of proteins (Bray, 1997).

Plants have evolved two major mechanisms to cope with water deficit: stress avoidance and stress tolerance (Ramajulu and Bartels, 2002). Stress avoidance may be the production of seeds before drought conditions prevail. But avoidance can also be achieved by specialised adaptations in the plant architecture. For example, the development of specialised cell surfaces to minimise loss of water by transpiration, an increase in root length, or a decrease in leaf surface area. On the other hand, water stress tolerance appears to be a combination of physiological and biochemical

alterations at the cellular and molecular levels. For example, the accumulation of various osmolytes and Late Embryogenesis Abundant (LEA) proteins and an efficient antioxidant system.

At present, hundreds of genes that are induced under drought have been identified, and a range of tools (gene expression patterns, transgenic plants, etc) is being used to study their functions in plant's adaptation to water deficits (Chaves et al., 2003). The products of those genes are thought to function not only in stress tolerance but also in the regulation of gene expression and signal transduction (Yamaguchi-Shinozaki et al., 2002; Fig. 1.3). However, because the responses to stress are complex, the functions of many of these genes are still unknown (Bray, 2002). Research in the sensing and signalling mechanisms of water deficit stress have also made dramatic progress (Bray, 1997; Shinozaki and Yamaguchi-Shinozaki, 1997). It is now clear that signalling pathways constitute a complex network, interconnected at many levels (Knight and Knight, 2001).

A major breakthrough in the understanding of water flow through living plant cells was the discovery of aquaporins (Tyerman et al., 2002). These can increase water permeability of cellular membranes by 10-20 fold and their expression and activity is modulated by dehydration. Several genes encoding aquaporins have been shown to be upregulated in *Arabidopsis thaliana* (Yamaguchi-Shinozaki et al., 1992) and in *Craterostigma platagenium* in response to drought or ABA (Ramanjulu and Bartels, 2002).

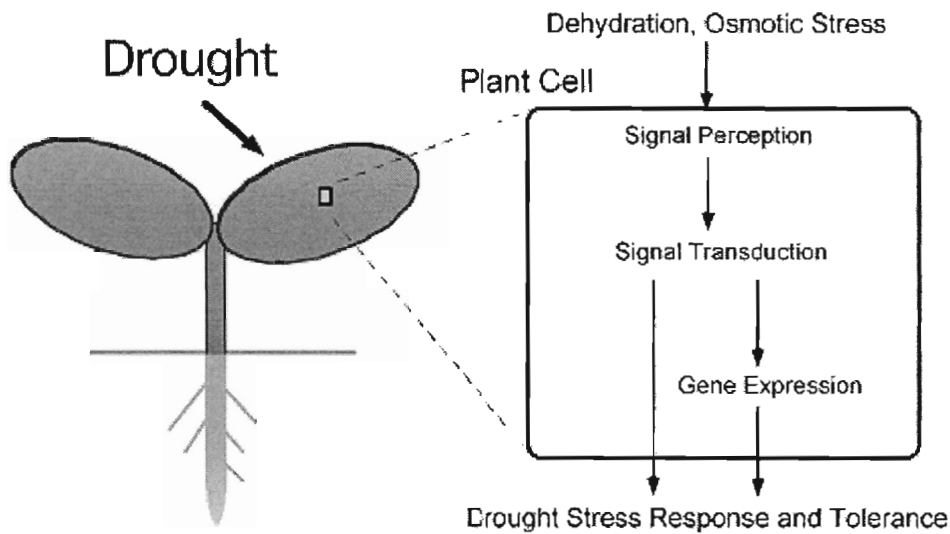


Figure 1.3. Schematic representation of molecular responses to drought stress in plant cells. Molecular and cellular responses to drought stress include perception of dehydration signal, signal transduction to cytoplasm and nucleus, gene expression, and responses and tolerance to drought stress (Yamaguchi-Shinozaki et al., 2002).

In their natural environment, plants can either be exposed to slowly developing water shortage (within days to weeks or months) or face short-term water deficits (hours to days) (Chaves et al., 2003). In the former case, plants can either escape dehydration by shortening their life cycle or acclimate and optimize their resource gain in the long term. In the latter case, plants react by minimising water loss or by developing metabolic protection against damage effects.

1.1.1 How do Plants Sense Stress?

The first step in the response to water deficit stress is the perception of the stress. Environmental signals are thought to be first perceived by specific receptors (Xiong and Zhu, 2001). These receptors, upon activation, will either initiate or suppress a cascade to transmit the signal intracellularly. Very often, nuclear transcription factors are activated to induce the expression of a specific set of genes. A common form of signal initiation is receptor-coupled protein phosphorylation. Current research indicates that receptor-like protein kinases, two-component histidine kinases and G-protein-associated receptors may represent the potential sensors of these signals. In *Arabidopsis*, a gene that encodes a receptor-like kinase with leucine-rich repeats, *RPK1*, was found to be induced one hour after ABA treatment, dehydration, high-salt

and low-temperature treatments (Hong et al., 1997). This protein kinase might therefore be involved in multiple-stress signal transduction. The best characterized two-component histidine kinase is the *Saccharomyces cerevisiae* osmosensor SNL1. SLN1 and the YPD1-SSK1 response regulator form the 'two-component' signal unit which regulates the high-osmolarity glycerol (HOG) MAPK cascade, resulting in the production of glycerol to survive osmotic stress. Using degenerate primers in PCR, an Arabidopsis histidine kinase gene, *AtHTK1*, was isolated, which is structurally related to SNL1 (Urao et al., 1999). In a complementation study, *AtHTK1* was found to rescue the salt sensitivity of yeast mutants with deletions of SNL1 and SHO1 (another trans-membrane osmosensor).

1.1.2 Signalling Events

Following the first perception of osmotic change during water stress, a signalling mechanism must be activated to induce specific genes (Bray, 1997). As discussed above, this involves protein phosphorylation and dephosphorylation (Lee et al, 1999). Many studies have suggested that changes in cytoplasmic Ca^{2+} concentration is involved in various intracellular signalling processes (Sanders et al., 1999; Xiong and Zhu, 2001). Cytosolic Ca^{2+} concentration is low, and upon stimulation, Ca^{2+} is released from intracellular storage or enters the cell via Ca^{2+} channels. In higher plants, the phosphoinositide module is involved in the transduction of environmental stimuli such as light, gravity and osmotic stress (Stevenson et al., 2000). The IP_3 receptor, which is required for the activation of store-operated Ca^{2+} channels, has a precursor: phosphatidylinositol 4,5-bisphosphate (PIP_2). This precursor is synthesized via phosphatidylinositol 4-phosphate 5-kinase. In Arabidopsis, a gene encoding this enzyme, *PIP5K*, was induced by water stress and ABA (Mikami et al., 1998).

Protein phosphorylation and dephosphorylation are the most common intracellular signalling modes (Hardie, 1999). They are involved in the regulation of a wide range of cellular processes, including enzyme activation, assembly of macromolecules, protein localization and degradation, but also in environmental stress responses. PKABA1, a serine/threonine protein kinase from wheat, was found to be upregulated by drought, low temperature, NaCl, as well as ABA stresses (Holappa and Walker-Simmons, 1995). Some of the most abundant regulatory protein kinases involved in abiotic stress signalling are Ca^{2+} -dependent (CDPK) (Knight and Knight, 2001). The

expressions of the Arabidopsis genes *AtCDPK1* and *AtCDPK2* were rapidly induced by dehydration and salt treatments.

In the signalling cascade, inactivation of the phosphoproteins is usually achieved by dephosphorylation (Xiong and Zhu, 2001). There are 4 major subgroups of protein phosphatases: PP1, PP2A, PP2B (calcineurin) and PP2C. The last two are Ca^{2+} -dependent while the first two are not. Studies using phosphatase inhibitors have indicated a role for phosphatases in stress signalling. In alfalfa plants, a phosphatase 2A inhibitor, okadaic acid, induced the expression of a cold-induced gene, *cas 15*, at 25°C. Several MAPKs and CDPKs have been identified in plants subjected to water stress and shown to be involved in transducing the dehydration signals sensed at the plasma membrane to the nucleus.

ABA signalling is very important to any discussion of stress responses. Osmotic stress leads to ABA accumulation. The Arabidopsis *abi1* and *abi2* are dominant mutants which show insensitivity to exogenous ABA application during germination (Leung et al., 1997). In these mutants, regulation of the stomata is affected, leading to a wilted phenotype in low humidity conditions. Previous studies based on these insensitive mutants and ABA-deficient mutants led to the consensus that there exist both ABA-dependent and ABA-independent stress signalling pathways. In the former, the accumulation of ABA activates various stress-associated genes, some of them being recognised as stress adaptive (Chaves et al., 2003). The product of these genes are either functional (such as aquaporins or the enzyme of osmoprotectant biosynthesis) or regulatory (such as protein kinases). As shown in Figure 1.4, it is now hypothesized that at least four independent signal transduction pathways function in the activation of the stress-inducible genes under dehydration conditions: two are ABA-dependent (pathways I and II) and two are ABA-independent (pathways III and IV) (Yamaguchi-Shinozaki et al., 2002).

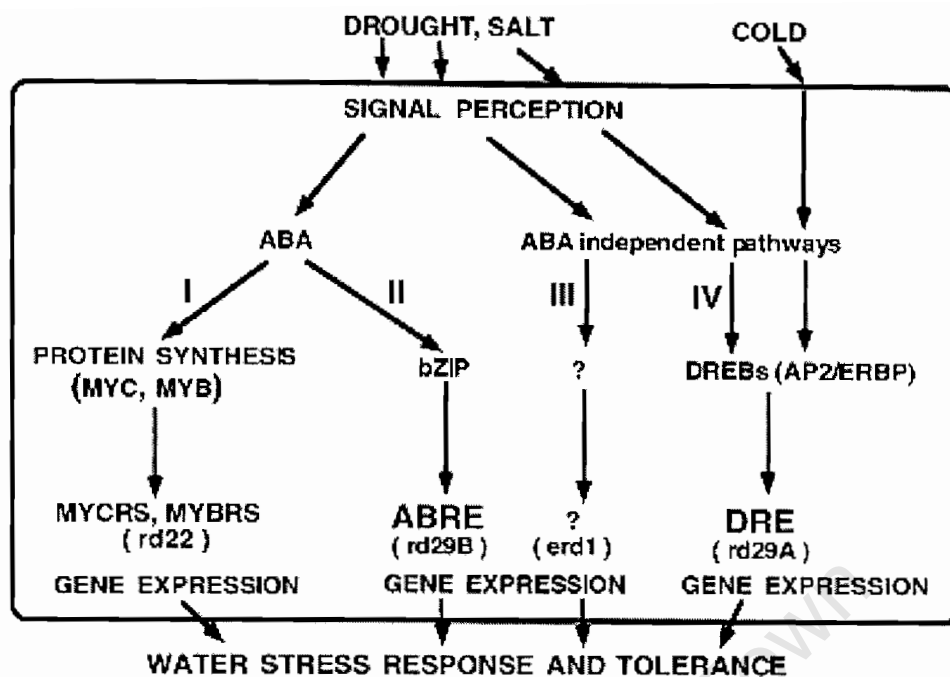


Figure 1.4. Signal transduction pathways between initial dehydration stress signal and gene expression. There are at least four signal transduction pathways: two are ABA-dependent (I and II) and two are ABA-independent (III and IV). Protein synthesis is necessary for one of the ABA-dependent signal pathways (I). ABRE (ABA response element) is involved in one of the ABA-dependent pathway (II). In one of the ABA-independent pathways, DRE (dehydration response element) is involved in the regulation of genes not only by drought and salt but also by cold stress (IV). Another ABA-independent pathway is controlled by drought and salt, but not by cold (III) (Yamaguchi-Shinozaki et al., 2002).

1.1.3 ABA-mediated Signalling

The accumulation of ABA under water stress may be as a result of enhanced biosynthesis and/or a decrease in breakdown (Bray, 1997). ABA biosynthesis starts at the epoxidation of zeaxanthin by zeaxanthin epoxidase (ZEP) to form epoxyxanthophyll precursors (Taylor et al., 2000). In both roots and leaves, the key regulatory step for ABA synthesis is catalysed by 9-*cis*-epoxycarotenoid dioxygenase (NCED) (Qin and Zeevaart, 1999). This enzyme converts the epoxycarotenoid precursor to xanthoxin in the plastids. Xanthoxin is then converted to ABA by two cytosolic enzymes via abscisic aldehyde. The *NCED* gene has been cloned from cowpea, tomato, beans and maize, and has been shown to have a promoter that is induced by dehydration in roots and leaves (Taylor et al., 2000; Thompson et al., 2000).

ABA induces the expression of several genes. *Cis*- and *trans*-acting factors have been defined that are involved in the transcriptional regulation of genes by ABA (Bray, 2002), as well as protein kinases or phosphatases interacting with Ca^{2+} (Bowler and Fluhr, 2000). Several genes that are upregulated under dehydration stress conditions contain a conserved ABA-responsive element (ABRE) in their promoter regions. This element has a slightly different DNA sequence in different genes. They have a core ACGT-containing G-box and are bound by *cis*-acting basic leucine zipper (b-ZIP) transcription factors (Fig. 1.4). They bind as dimers, and two elements are required for a response (Bray, 2002). Several b-ZIP factors (ABRE1-3) have been cloned from *Arabidopsis*, and their transcription has been found to be upregulated by drought and salt stress (Uno et al., 2000).

There are *cis*-acting elements other than ABRE that function in ABA-responsive gene expression (Shinozaki and Yamaguchi-Shinozaki, 1997). Two of these, the MYC-like and MYB-like elements, which have been defined in *Arabidopsis*, are involved in regulating the expression of ABA-induced genes in response to severe water deficit (Fig. 1.4; Bray, 2002). These two elements (MYC: ACACATGT and MYB: YAAC(G/T)G) were defined in the dehydration-responsive gene *RD22* (Abe et al., 1997). A cDNA for a MYC homologue named *rd22BP1* was cloned and the gene is induced by dehydration and salt stress. In *Arabidopsis*, high-salt concentrations and exogenous application of ABA result in the induction of *Atmyb2*, a MYB-related protein.

1.1.4 The Roles of ABA

One of the main effects of ABA at the leaf level was found to be stomatal closure (Wilkinson and Davies, 2002). However this statement is now under debate. Recently, Holbrook et al., (2002) grafted tomato plants constructed from the ABA-deficient tomato mutants *sitiens* and *flacca* and their near isogenic wild-type parents, and demonstrated that stomatal closure in response to soil drying can occur in the absence of leaf water deficits and may not require ABA production in the roots. The effects of ABA on stomatal closure may be mediated by a chemical signal originating in the roots (other than ABA itself) that may then lead to changes in apoplastic ABA levels in leaves, which may then cause stomatal closure. The induction of stomatal closure in leaves by apoplastic ABA under water stress is mediated by phospholipase D

activity and by the hyperpolarisation of membrane Ca^{2+} channels and tonoplast K^{+} channels of the guard cells (MacRobbie, 2000).

Roots play a very important role in sensing soil water deficits (Wilkinson and Davies, 2002). Sustained root growth under moderate levels of water stress, as opposed to inhibition of root growth in the early phases of drought, results from rapid osmotic adjustment. This allows for partial turgor recovery and maintenance of the ability to loosen cell walls (Hsiao and Xu, 2000). The ability of roots to sustain growth under mild water stress is correlated with ABA accumulation. Maize mutants deficient in ABA production maintained better shoot growth than root growth at low water potentials, as compared to the wild type (Wilkinson and Davies, 2002).

Contrary to roots, leaf growth is severely inhibited during drought. Changes in pH regulate leaf expansion during water stress (Hsiao and Xu, 2000), and inhibition of growth is mediated by a rapid decrease in extensibility of expanding leaf cell walls. These changes are associated with the inhibition of wall acidification, which might occur due to the inactivation of plasma membrane H^{+} -ATPases under water stress (Bogoslavsky and Neumann, 1998). The local expression of expansins induces the process of leaf development and modified leaf shape (Pien et al., 2001 cited in Chaves et al., 2003). However, it is not clear how water stress affects expansin accumulation. An increase in the xylem sap pH may act as a drought signal to reduce leaf expansion via an ABA-mediated mechanism, as found with ABA-deficient tomato and barley mutants (Bacon et al., 1998). Additionally, studies with ABA-deficient tomato mutants suggest that maintenance of leaf expansion requires endogenous ABA (Sharp et al., 2000). The impairment of leaf growth in these mutants caused by ABA deficiency was in part attributed to ethylene.

1.1.5 Non-ABA-mediated Signalling

Several genes are induced by dehydration and other stresses in ABA-deficient and ABA-insensitive mutants in *Arabidopsis*, which suggests that such genes do not require ABA for their expression (Fig. 1.4; Shinozaki and Yamaguchi-Shinozaki, 1997). Such ABA-independent genes have a conserved dehydration responsive element (DRE; TACCGACAT consensus) in their promoters. Two groups of transcription factors that bind to the *cis*-acting DRE element have been cloned in

Arabidopsis and characterized (Knight and Knight, 2001). The *drebl* (or *cbf1*) and *drebl2* (or *cbf2*) genes encode structurally different proteins that bind to the DRE element but are induced by different stresses. Low temperature and water deficit induce *cbf1* (Stockinger et al., 1997), and drought and osmotic stresses induce *drebl2* (Lui et al., 1998). Two isoforms of DREB2 have been cloned and they show differential tissue specificity. *Dreb2A* is root specific in response to salinity, whereas *drebl2B* is expressed in stems and roots in response to drought (Nakashima et al., 2000).

The sharing of ABA-dependent and ABA-independent pathways may also occur downstream of the first stress recognition and signalling events. Alternatively, a gene may contain both DRE and ABRE elements in its promoter. For example, in Arabidopsis, the regulation of the *rd29A* gene (which contains both elements) is independent of ABA in the first hours of dehydration, but becomes ABA-dependent in the later stages of expression (Shinozaki and Yamaguchi-Shinozaki, 2000).

1.1.6 Other Mechanisms

Due to either water stress or cold stress, membrane lipids have also been postulated to 'sense' osmotic stress by changing their fluidity (Knight and Knight, 2001). These changes affect ion and water channels. This mechanism involves phospholipase C and D as well as phosphatidyl-4,5-phosphatase 5-kinase (PIP5; Mikami et al., 1998 cited in Chaves et al., 2003). Frank et al. (2000) cloned the two phospholipase D genes (*CpPLD1* and 2) involved in early dehydration in the resurrection plant *C. plantagineum*. *CpPLD2* was induced specifically by dehydration and abscisic acid.

Previously, ethylene was mostly studied in connection with hypoxia, for example during flooding. However, there were some suggestions for a role of ethylene during dehydration (Feng and Baker, 1992; Michelozzi et al., 1995). Recent evidence suggests that ABA influences growth by mediating ethylene effects in plant organs. Ethylene-response factors (ERFs) have been cloned in Arabidopsis and tobacco and shown to mediate ethylene-induced signalling cascades (Solano et al., 1998). In Arabidopsis, a set of transcriptional activators and repressors were cloned (*AtERF1-5*) that bind to the ERE (Fujimoto et al., 2000). The *AtERF4* gene was induced by

dehydration stress and the *AtERF3* gene was moderately induced by drought and salinity, apparently independent of ABA and ethylene.

1.2 FUNCTIONS OF DROUGHT INDUCIBLE GENES

The products of drought inducible genes in various species have been predicted from sequence homology with known proteins. Many of those genes are not only drought inducible, but also induced by salt and low temperature stresses (Yamaguchi-Shinozaki et al., 2002). The products of those genes are thought not only to protect cells from water deficit by production of important metabolic proteins, but also in the regulation of genes for signal transduction. As shown in Figure 1.5, these genes are classified into two groups. The first group includes proteins that probably function in stress tolerance. These are chaperones, LEA proteins, osmotins, antifreeze proteins, mRNA binding proteins, water channel proteins, sugars and proline transporters, etc. The second group contains protein factors involved in further regulation of signal transduction and gene expression such as protein kinases, transcription factors and enzymes in phospholipids metabolism.

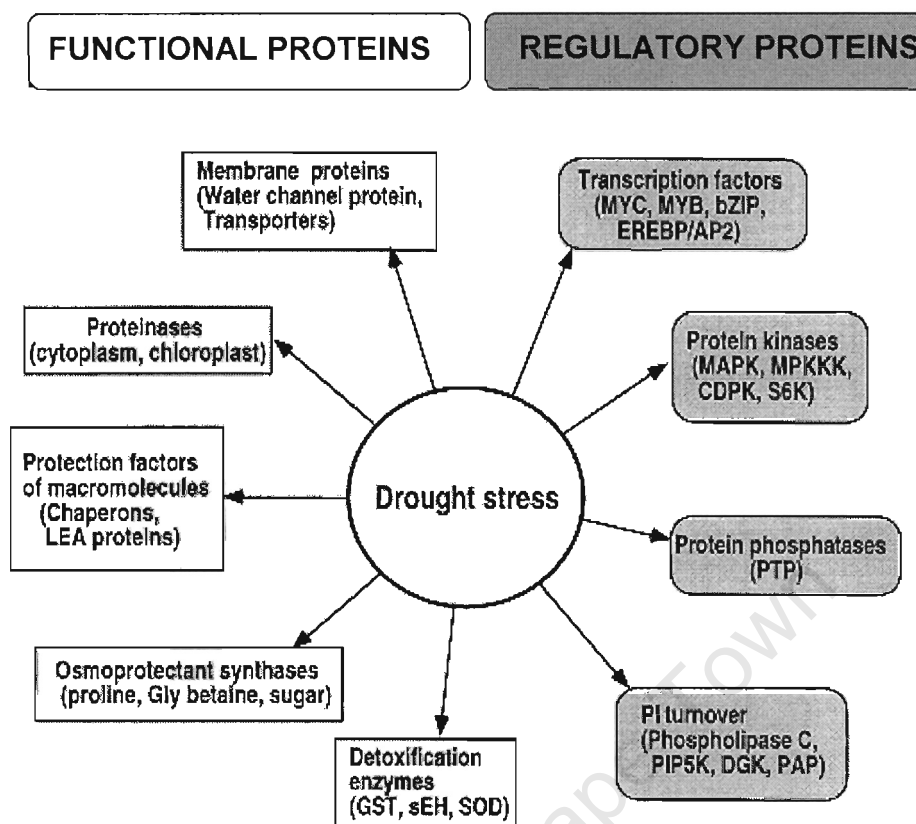


Figure 1.5. Drought inducible genes and their possible functions in stress tolerance and response. Gene products are classified into two groups. The first group includes proteins that probably function in stress tolerance (Functional proteins), and the second group contains protein factors involved in further regulation of signal transduction and gene expression that probably function in stress response (Regulatory proteins) (Yamaguchi-Shinozaki et al., 2002).

1.2.1 LEA Proteins

As their name suggests, LEA proteins are those that increase in abundance in seeds during the latter stages of seed development. They also accumulate in response to dehydration, low temperature, salinity, or exogenous ABA application (Ramanjulu and Bartels, 2002). They accumulate in resurrection plants in response to drought stress, as like the majority of seeds, resurrection plants are desiccation tolerant (Swayze, 2004). LEA proteins are widely distributed among monocot and dicot species, and many different forms have been isolated. Their homology to each other, their conserved protein domains, their ubiquity and their expression patterns implies a fundamental role in desiccation tolerance. It has been proposed that they may play a

role in the protection of cytoplasmic structures during dehydration (Ramanjulu and Bartels, 2002). LEA proteins have been divided into groups based on their predicted biochemical properties and protein motifs (Table 1.1). Heat soluble and hydrophilic LEAs are primarily located in the cytoplasm and nuclei of cells (Roberts et al., 1993). Some LEAs are thought to coat intracellular macromolecules with a cohesive water layer as a sort of preferential hydration (Dure, 1997). As dehydration progresses, LEAs provide a layer of their own hydroxylated residues to interact with the surface group of other proteins, acting as 'replacement water'. It is postulated that some LEA proteins have the ability to preserve cellular macromolecules in a similar manner to sucrose. However, the mechanisms behind this protection are not yet fully understood (Swayze, 2004). As a result of introducing the LEA protein gene *HVA1* from barley into rice, *HVA1* protein accumulated in leaves and roots of the rice plants. Second-generation transgenic rice plants showed significantly increased tolerance to water deficit and salinity (Datta, 2002). A maize lea protein, DHN1, which has at least one copy of a consensus 15-amino acid sequence, the 'K segment' (koag et al., 2003). This segment is highly conserved in all plants and resembles a lipid binding class. The maize DHN1 was found to bind lipid vesicles that contain acidic phospholipids. This association results in an apparent increase in α -helicity of the protein. It is therefore postulated that such LEAs undergo function-related conformational changes at the water/membrane intersurface. This might be related to the stabilization of vesicles or other endomembrane structures under stress conditions.

Table 1.1. Characteristics of five sub-groups of LEA proteins (Swayze, 2004).

Sub-Group	Characteristics
Group 1	Group 1 LEAs are extremely hydrophilic and contain a high proportion of glycine, glutamate and glutamine residues. They share a 20 amino-acid motif that may be repeated within the protein
Group 2	Also known as dehydrins or the D-11 family, group 2 LEAs commonly contain lysine-rich 15 amino-acid motifs known as K-segments. They may act as structural stabilisers and are thought to have chaperone properties. Dehydrins have been found to operate in both the cytoplasm and the nucleus and are the best studied of the five LEA sub-groups
Group 3	Group 3 LEA proteins are distinguished by an 11 amino-acid motif that is thought to form an α -helix structure
Group 4	These LEAs share conserved N-sequences that are also thought to form α -helices
Group 5	Because they contain many hydrophobic residues, group 5 LEAs probably form globular structures in plant cells

1.2.2 Heat Shock Proteins

Heat Shock Proteins (HSPs), which are sometimes called ‘stress proteins’, are also induced under water stress. HSPs vary greatly in size, but small HSPs (sHSP) are thought to be most relevant in desiccation tolerance (Waters et al., 1996). They range in size from 15 to 30 kDa, and up to 30 unique sHSPs have been found to accumulate in response to heat stress. In desiccation-sensitive seeds of *Arabidopsis*, their expression is much reduced (Wehmeyer and Vierling, 2000). Because of little tissue specificity, they are thought to have an overall protective effect during drying. They might act as molecular chaperones. Generally, HSPs are able to maintain partner proteins in a folding competent, folded or unfolded state, so as to minimize the aggregation of non-native proteins, or to target non-native or aggregated proteins for degradation and removal from the cell (Feder and Hofmann, 1999). Recently the HSP12 protein from yeast was found to be associated with membranes in desiccated yeast cells.

1.2.3 Aquaporins

Aquaporins are essential components in cellular water transport throughout the plant cell (Maurel and Chrispeels, 2001). They are a complex family of channel proteins that transport water along trans-membrane water potential gradients. They can control the hydraulic conductivity of membranes and hence increase water permeability 10-20 fold. The regulation of their expression and activity is modulated by dehydration. For example, the *rd28* gene from *Arabidopsis* and the tomato-ripening-associated membrane protein (TRAMP) are both upregulated by dehydration (Yamaguchi-Shinozaki et al., 1992; Fray et al., 1994). In the resurrection plant *C. plantagineum*, several aquaporins were found to be upregulated by dehydration (Mariaux et al., 1998). Some are inducible by both dehydration and ABA, while others are inducible by drought only, suggesting the involvement of ABA-dependent and -independent signalling pathways.

1.2.4 Osmoprotectants

Osmoprotectants, or compatible solutes, are synthesized in response to osmotic stress. They are called compatible because they do not interfere with cellular structure and function. Their accumulation during water deficit is termed osmotic adjustment (Yamaguchi-Shinozaki et al., 2002). Because of increased solute concentrations in the plant cell, water loss is inhibited and the adverse effects of water stress are reduced. Osmolytes include sugars, polyols, proline, quaternary ammonium compounds and tertiary sulfonium compounds (Ramanjulu and Bartels, 2002).

Glycine betaine: This is one of the most effective osmoprotectants (Hanson, 1998). It occurs naturally in some crop plants such as sugar beet and cotton, but also stress-tolerant wild plants. However, more susceptible crops do not contain significant amounts of the compound. The pathway of glycine betaine synthesis has been identified. Introduction of the genes coding for the enzymes in the pathway in tobacco resulted in only a small accumulation of glycine betaine and no significant tolerance against water or salt stress.

Trehalose: This disaccharide helps maintain individual cell structure and function during severe environmental stress conditions (Wu and Garg, 2003). Trehalose acts as a water substitute on the surface of macromolecules which are hence protected against

desiccation. Introduction of genes from yeast or *Escherichia coli* that encode enzymes for trehalose synthesis into tobacco and potatoes resulted in enhanced trehalose levels and drought tolerance (Penna, 2003). However under normal conditions, the plants had stunted growth and other unwanted characteristics. With the use of stress-inducible promoters, this problem was solved. There are many indications that trehalose protects the plants not only during water-deficit stress but also during freezing and against high salt concentrations. In addition, engineering trehalose biosynthesis in crop plants may possibly confer improved storage properties after harvest (Holmström et al., 1996).

1.2.5 Antioxidant Signalling

Low molecular weight antioxidants, such as ascorbate, glutathione and tocopherol, have crucial roles in defence and acting as enzyme co-factors (Foyer and Noctor, 2005a). Additionally, such cellular antioxidants affect plant growth and development by modulating such processes as mitosis, cell elongation, senescence and cell death (De Pinto and De Gara, 2004; Potters et al., 2004). They also provide essential information on cellular redox state which influences gene expression associated with biotic and abiotic stress responses to maximise defence. Reactive Oxygen Species (ROS) such as the hydroxyl radical ($\cdot\text{OH}$) or the superoxide anion ($\cdot\text{O}_2^-$) are natural products of the photosynthetic process. Under normal conditions, such unstable ions exist only transiently. However, under conditions of osmotic stress, ROS are over-produced, causing extensive cellular damage and inhibition of photosynthesis. This is called oxidative stress. Plants have evolved systems to combat oxidative stress. Various enzymes aid in reducing the active oxygen species in order to protect the plant cells from damage (Yamaguchi-Shinozaki et al., 2002).

Numerous enzyme systems produce superoxide or H_2O_2 . Because of their reactive nature, the accumulation of these intermediates must be controlled. The extent of the accumulation of ROS is determined by the antioxidative system, enabling organisms to maintain proteins and other cellular components in an active state for metabolism. Plant cells synthesize high levels of ascorbate (vitamin C) which provides high protection against oxidative challenge (Foyer et al., 2005). Plants also make glutathione (GSH), which maintains most cytoplasmic thiols in a reduced ($-\text{SH}$) state,

and tocopherols (vitamin E), which is considered to be a major singlet oxygen scavenger and other ROS. In many reactions involving GSH, the Cys thiol group is oxidised to yield glutathione disulphide (GSSG), and the reverse reaction is catalysed by glutathione reductase (GR) using NADPH (Noctor et al., 2002). Millimolar concentrations of GSH act as a key redox buffer, forming a barrier between protein Cys groups and ROS. GSH is also a substrate for enzymes that reduce peroxides (Foyer and Noctor, 2005b). Glutathione-S-transferases (GSTs) constitute a large family of proteins with a large range of functions. Genes of glutathione peroxidase (GPX) are strongly induced by ROS (Levine et al., 1994). However, compared to catalases, ascorbate peroxidases and peroxiredoxins, GPXs probably make a small contribution to overall peroxide metabolism.

Another important antioxidant is superoxide dismutase (SOD) which converts two superoxide anions into a molecule of hydrogen peroxide and one of oxygen. Catalase and ascorbate peroxidases catalyse the decomposition of hydrogen peroxide into oxygen and water (Scandalios et al., 1997).

The modulation of the ROS-antioxidant interaction plays an important part in the regulation of plant development as well as responses to stresses (Foyer and Noctor, 2005a). The symbiotic association between organisms also involves ROS-antioxidant interactions, leading to the enhancement of antioxidant status, and this symbiotic partnership can be more resistant to environmental stress than either partner alone (Kranter et al., 2005).

Tobacco plants transformed with the genes encoding ascorbate peroxidase and glutathione peroxidase, both enzymes presumed to have a role in mopping up peroxides in the plant cell, maintained almost near normal rates of photosynthesis under stress conditions while photosynthesis in non-altered plants was reduced by half (Moffat, 2002).

The apoplast is the site where oxidants are produced and perceived (Pignocchi et al., 2003). Unlike the cytoplasm, the apoplast is deficient in NAD(P)H and glutathione and has more oxidised ascorbate than the cytoplasm. The redox buffering capacity of the apoplast is much weaker than that of the cytoplasm. Current knowledge of redox

controls in the apoplast and the cytoplasm predicts that (1) the plasma membrane acts as an important site for the perception and transduction of environment change through redox signals; (2) interactions between receptor protein containing oxidizable thiols in the membrane surface are facilitated by apoplastic redox changes; (3) due to the low redox buffering capacity of the apoplast, a steep redox gradient is present across the plasma membrane; (4) such a gradient triggers membrane channels activity, involving calcium release and aquaporin function. Therefore, antioxidants function as key signalling compounds that constitute a dynamic metabolic interface between plant cell stress perception and physiological responses.

Over the past years, several other studies have been conducted on detoxification enzymes. Peroxiredoxins (Prxs) have received considerable attention as a new and expanding family of thiol-specific antioxidant enzymes (Chae et al., 1994). This particular family of antioxidant enzyme will be discussed in detail at a later stage in this chapter.

1.2.6 Transcription Factors

Transcription factors are small DNA binding molecules that attach to specific sites on a DNA strand in order to activate or deactivate the expression of certain genes. Two cDNA clones that encode DRE binding proteins, *dreb1a* and *dreb2a*, were isolated by the yeast one-hybrid technique (Lui et al., 1998). Transgenic Arabidopsis plants constitutively over-expressing either gene showed growth retardation phenotypes under normal growth conditions. However, over-expression of the DREBs strongly induced the stress-inducible genes *kin1*, *cor6.6/kin2*, *cor15a*, *cor47/rd17* and *erd10* genes, as well as the *rd29A* gene (Shinozaki and Yamaguchi-Shinozaki, 2000).

1.3 RESURRECTION PLANTS

Resurrection plants are a group of plants which possess a unique and effective mechanism for coping with drought stress by being desiccation tolerant (Sherwin and Farrant, 1996). They are mostly native to areas of arid climate in the world such as southern Africa, southern America, and Western Australia (Gaff, 1977; 1987). Resurrection plants can survive the loss of most of their tissue water content, usually down to 5% relative water content (RWC) until a quiescent stage is achieved. Upon rewatering they revive rapidly and are restored to their hydrated state. There are two

main categories of vegetative desiccation tolerant plants: fully desiccated that can withstand the total loss of free protoplasmic water at any rate and modified desiccation tolerant plants that can only survive if water loss is slow (Oliver et al., 1998). Desiccation in resurrection plants prevents growth and reproduction over the dehydrated period (Scott, 2000). Tissue damage appears to be minimal to non-existent and the plant is preserved until water becomes available.

Resurrection plant species are represented in most taxonomic groups, ranging from pteridophytes to dicotyledons (Hartung et al., 1998). These include terrestrial species such as the monocotyledonous *Xerophyta viscosa* (Baker), the dicotyledonous shrub *Myrothamnus flabellifolia* (Welw.), and the aquatic species *Chamaegigas intrepidus* (Dinter). The terrestrial species are often found as rocky outcrops in shallow sandy soil (Sherwin and Farrant, 1995). The ability to withstand such water loss is common to some algae and lichens, and in some liverworts and mosses, some fern-like species and some ferns (Bonhert, 2000). Angiosperm resurrection plants have been subdivided into two groups: homiochlorophyllous plants, which retain their chlorophyll during drying (such as *C. plantagineum*), and poikilochlorophyllous plants, which lose chlorophyll on drying (such as *X. viscosa*). In *X. viscosa*, the loss of chlorophyll in the leaves is accompanied by the accumulation of anthocyanins (Sherwin and Farrant, 1998). Dismantling of the photosynthetic apparatus and production of anthocyanins are thought to protect the plant against UV-light damage and free radical damage.

One of the most thoroughly investigated metabolic changes in resurrection plants as they dehydrate and rehydrate is the accumulation of carbohydrates. Sucrose is the dominant carbohydrate to accumulate (Scott, 2000). It is thought to protect the dehydrated cell, firstly by stabilizing the membranes and proteins in the dry state by maintaining hydrogen bonding within and between macromolecules (Allison et al., 1999). Secondly the sugars could be involved in vitrification of the cell contents and thus stabilize internal cell structures. Furthermore, in experiments where isolated enzymes have been dried in the presence of sucrose, there is good evidence that the enzymes remain stable in the dried state (Suzuki et al., 1997). Sucrose has also been shown to preserve the integrity of the lipid bilayer during desiccation.

Ingram and Bartels (1996) isolated several genes from *C. plantagineum* which possess homology with LEA proteins. Since many seeds have a degree of desiccation tolerance, the presence of LEAs in resurrection plants is not surprising. However their exact role in desiccation is still not known, but they are likely to be of structural importance, associating with internal structures of cells to stabilize them. In the resurrection plant *X. viscosa*, Mundree and Farrant (2000) have shown that in response to dehydration, instead of losing water and shrinking, the central vacuole subdivides into a number of smaller vacuolar structures. It has been hypothesized that these structures become filled with compatible solutes such as ions, small proteins and sugars. Because of their unique ability to withstand desiccation, resurrection plants are valuable in our study of the mechanisms involved in achieving desiccation tolerance in plants. In this particular study, genes from a *Xerophyta* species, *X. viscosa*, is being investigated. It is a monocotyledonous plant of the velloziaceae family, indigenous to arid regions of southern Africa, such as Cathedral peak in the Drakensberg mountains. The ‘stems’ of *X. viscosa* are crowned by an apical tuft of leaves, and are in fact pseudostems. The actual stem is only a few millimetres in diameter and is enclosed in a dense sheath of adventitious roots and leaves (<http://www.biologie.uni-rostock.de/abt/botanik/inselbergs/en-oeko.html>). We have observed that the plants flower mainly after rehydration following a period of almost complete dehydration.

1.4 PEROXIREDOXINS

Peroxiredoxins (Prxs), also termed the thiol-redoxin peroxidases and alkyl-hydroperoxide-reductase-C22 proteins, have received considerable attention in recent years (Chae et al., 1994). This family of peroxidases is found in all organisms and is unrelated to any other peroxidase family (Hoffman et al., 2002). Prxs exert protection as an antioxidant by their peroxidase activity ($\text{ROOH} + 2\text{e}^- \rightarrow \text{ROH} + \text{H}_2\text{O}$), where hydrogen peroxide, peroxyxynitrite and a wide range of organic hydroperoxides (ROOH) are reduced and detoxified. The first identified Prx, the thiol-specific antioxidant protein (TSA), was shown to protect glutamine synthase activity and DNA integrity in a mixed-function oxidation (MFO) system (Chae et al., 1994; Kim et al., 1988). Four groups of Prxs exist: the 2-Cys Prx, which has been mostly studied.

The 1-Cys Prx and type II Prx, both found in most organisms including mammals, and the Prx Q group, found in bacteria and plants but not in animals (Seo et al., 2000).

1.4.1 The Family of Plant Prxs

A search of the Arabidopsis genome has identified members of all four Prx families in a single plant with a total of 10 open reading frames (ORFs) (Table 1.2) (Horling et al., 2002; Dietz KJ, 2003). They are all nuclear encoded proteins.

1-Cys Prx: This was the first type of Prx identified in plants and was found to be expressed in the embryo and aleurone layer of developing barley seeds (Aalen et al., 1994; Stacy et al., 1999). It is named as such as it has only one cysteine residue in its catalytic domain. The rice orthologue was also identified in dormant seeds, and the *1-Cys Prx* transcript rapidly decreased to low levels as germination proceeded (Lee et al., 2000). Tobacco leaves in which the rice 1-Cys Prx was over-expressed were less susceptible to H₂O₂-mediated oxidative damage. This supports the hypothesis that 1-Cys Prx protects the embryo and the aleurone layer from oxidative damage during desiccation of the seed. The protein is nuclear localized, which means that it might protect DNA and the nuclear machinery of transcription (Stacy et al., 1999). The yeast and the barley 1-Cys Prx were found to protect DNA from nicking in an MFO DNA protection assay (Dietz, 2003). Although Table 1.2 states that the Arabidopsis 1-Cys (AtPer1) is nuclear localized, this has not yet been confirmed (Haslekas et al., 2003a).

2-Cys Prx: The first 2-Cys Prx was also isolated from barley (Baier and Dietz, 1996). It had high sequence similarity with the human and yeast thioredoxin-dependent peroxidase Tpx. The 2-Cys Prx was chloroplast located and two catalytic Cys residues are present at positions 36 and 158 in the amino acid sequence (Baier and Dietz, 1997). The 2-Cys Prx functions as a homodimer with a head-to-tail arrangement. The barley 2-Cys Prx complemented peroxide sensitivity of AhpC-deficient *E. coli*. Heterologously expressed 2-Cys Prx reduced H₂O₂, cumene hydroperoxide (CM-OOH), *tert*-butylhydroperoxide (tBOOH) and other hydroperoxides (König et al., 2002; König and Dietz, unpublished).

Table 1.2 Peroxiredoxins in the genome of Arabidopsis. Due to annotation errors, *prx II D* was only recognized recently as typical *prx*-encoding ORF and its expression will have to be investigated. The third column gives the amino acid length of the pre-protein and the predicted targeting address, respectively; the sixth column gives the position of the conserved Cys residues in the mature protein (adapted from Dietz KJ, 2003).

	MIPS A.th. DB #	aa/tp	kDa	IEP	Position of Cys-residues	Subcellular location
1-Cys Prx	At1g48130	216/-	24.1	6.14	46	Nucleus
2-Cys Prx						
A	At3g11630	266/83	29.1	4.91*	36, 158	Chloroplast
B	At5g06290	271/88	29.6	4.71*	36, 158	Chloroplast
Prx Q	At3g26060	216/57	23.7	5.53*	54, 59	Chloroplast
type II Prx						
A*	At1g65990	553	62.7	6.04	51, 76	Pseudogene (?)
B	At1g65980	162	17.4	5.17	51, 76	Cytosol
C	At1g65970	162	17.4	5.33	51, 76	Cytosol
D	At1g60740	174	19.2	6.1	51, 76	Cytosol
E	At3g52960	234/70	24.7	5.03*	51, 76	Chloroplast
F	At3g06050	199/28	21.2	6.29*	59, 84	Mitochondrion

*IEP calculated without transit sequence.

Type II Prx: This group of Prx was first identified in both yeast and Chinese cabbage (*Brassica campestris*) (Jeong et al., 1999; Dietz, 2003). The latter is a small protein (17.5 kDa) and has two Cys residues (positions 51 and 76). This protein can protect glutamine synthase from oxidative inactivation and also has H₂O₂ reduction activity (Choi et al., 1999). This activity was also shown for the Arabidopsis type II Prx (Horling et al., 2002). In the latter's genome, six genes with similarity to type II Prx were identified, four being expressed in leaves.

Prx Q: The fourth and last group of Prx was most recently cloned from plants (Kong et al., 2000). This protein (17 kDa) showed similarity to the bacterioferritin comigratory protein (Bcp) from *E. coli*. In Arabidopsis, one *prx Q* gene has been identified (Dietz et al., 2002). The active Prx Q is a monomer and reduces H₂O₂, tBOOH, and CM-OOH and is generated by thioredoxin. *E. coli* without the *bcp* gene is highly sensitive to tBOOH and CM-OOH.

1.4.2 The Mechanisms of Detoxification of Prxs

Prxs contain one or two Cys-residues, and function as monomers or dimers. The reaction mechanism comprises three steps: oxidation, derivatization, and regeneration of ground state (Hofmann et al., 2002). One specific cysteinyl residue reacts with the peroxide as the primary catalytic centre and is oxidised to sulfenic acid (reaction 1 in Fig. 1.6; Dietz, 2003). Sulfenic derivatives of cysteine react with other thiols to form disulfide bridges. Subsequent reactions depend on the Prx specific group. In 2-Cys Prx, Prx Q and type II Prx, a second sulfhydryl group of the Prx attacks the sulfenic acid group and a disulfide bridge is formed. H₂O is liberated (reaction 2 in Fig. 1.6). Formation of an inter- and intra-molecular disulfide bridge for 2-Cys Prx and the human type II Prx has been verified. In 2-Cys Prx, the second sulfhydryl group is located on the other subunit of the dimer, forming covalently-linked dimers (König et al., 2002). In type II Prx and Prx Q, both cyteines are located in the same polypeptide. The disulfide is regenerated by interaction with dithiols such as thioredoxin, glutaredoxin, etc (reaction 3 in right-hand branch of Fig. 1.6). The right-hand branch of the reaction sequence in Figure 1.6 represents the general catalytic cycle of Prx-mediated peroxide detoxification (Dietz, 2003).

A modified mechanism is required to explain the features of 1-Cys Prx. Only one Cys residue is available to react with the peroxide to form the sulfenic acid group. The oxidized 1-Cys Prx is regenerated in vitro by dithiothretol (DTT) (Choi et al., 1998). The physiological reductant is still unknown. Subsequently, a second Cys residue attacks the intermolecular disulfide bridge to form an intra-molecular disulfide bridge, thereby regenerating the functional Cys-residue of the Prx. Prx may either function in housekeeping antioxidant metabolism or be involved in oxidative stress resulting from environmental changes. In Arabidopsis, divergent light-, ascorbate-, and oxidative stress-dependent regulation of transcripts of the peroxiredoxin family suggest both housekeeping and stress-related functions (Dietz, 2003). Antisense mutants of Arabidopsis show a developmental retardation and metabolic phenotype with altered state of antioxidant system (Baier and Dietz, 1999). Under unfavourable growth conditions, early development was inhibited. The abundance of some photosynthetic proteins was reduced, and photosynthetic efficiency was reduced as well as chlorophyll content. The ascorbate pool was more oxidized in the 2-Cys Prx mutants,

which indicates that 2-Cys Prx is involved in antioxidant defence of photosynthetic organisms that is independent of the ascorbate system.

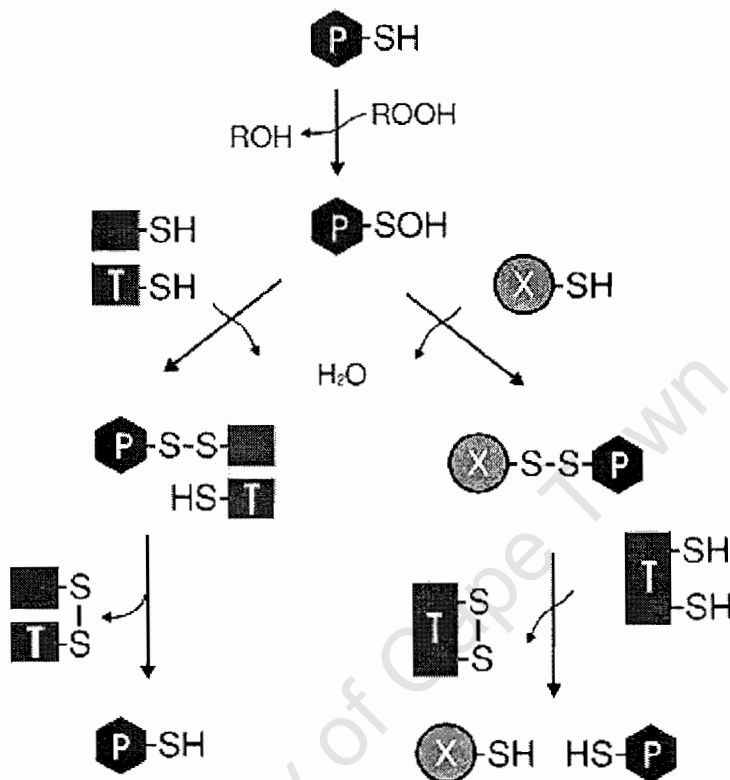


Figure 1.6. Generalized reaction mechanism of peroxiredoxin-mediated peroxide detoxification and regeneration of functional Prx. P: Prx protein environment of the primary reactive Cys residue. X: Environment of the second Cys residue which may be present in the same polypeptide chain either close to or distant from the first Cys residue. It may also be located in another polypeptide. T: Dithiol protein for reductive regeneration of Prx (Dietz, 2003).

1.5 IMPROVING STRESS TOLERANCE IN PLANTS

Rapid advances in the genetic engineering of plants have resulted from the study of the plant genome from the *Arabidopsis* model system (Cassells and Doyle, 2003). In contrast to traditional breeding and marker-assisted selection, the direct introduction of one or more genes by genetic engineering seems to be a more attractive and rapid method for improving stress tolerance in plants (Cushman and Bohnert, 2000). The

use of mutation techniques in *Arabidopsis* to obtain knock-out and up-regulated mutants have contributed greatly to genetic engineering.

Presently, the main engineering strategy relies on the transfer of one or several genes that encode either biochemical pathways or endpoints of signalling pathways that are controlled by a constitutively active promoter. Approaches already reported are the over-expression of biosynthetic enzymes for osmoprotectants (Nuccio et al., 1998), scavengers of ROS (Noctor and Foyer, 1998) and stress-induced proteins or LEA proteins (Thomashaw, 1999). It was recently demonstrated that the moderate over-expression of an orthologous cDNA encoding a sodium/proton antiporter can improve the salinity tolerance of *Arabidopsis* (Apse et al., 1999). 'Regulon' engineering, with the transformation of stress-specific transcription factors, which control the expression of a set of stress proteins, has been used to improve salinity, dehydration and freezing tolerance (Jaglo-Ottesen et al., 1998; Kasuga et al., 1999). However, the successes of these approaches are often limited by the lack of understanding of the metabolic flux, compartmentation and function. Therefore a deeper understanding of the complexity and the cross-talk of desiccation and temperature tolerance mechanisms is needed.

In the last decade, there has been a very rapid discovery of genes by the large-scale partial sequencing of randomly selected cDNA clones or expressed sequence tags (ESTs). Already for *Arabidopsis*, an extensive EST collection exists (Bevan et al., 1999), as well as for rice (Goff, 1999). EST initiatives for other crop species are well under way, for example, cotton, *Medicago truncatula*, maize, soybean, tomato and sorghum (www.nsf.gov/bio/pubs/awards/genome99.htm). Investigation of gene expression in stress-tolerant models is also underway. Such organisms have evolved additional structural and regulatory defences to allow them to withstand severe osmotic or ionic stresses and are therefore very useful to study.

Major EST sequencing has been performed for the halophyte *Mesembryanthemum crystallinum* and the halotolerant green alga *Dunaliella salina* (Gohkman et al., 1998). A comparison of ESTs between well-watered and salinity stressed *M. crystallinum* revealed that ~15% more genes were expressed in stressed than in unstressed plants (Cushman et al., 1999). The sequencing of 152 ESTs from a library of a desiccated

bryophyte resurrection moss, *Tortula ruralis*, showed that 70% of the ESTs represented novel sequences (Wood et al., 1999). Using differential, subtractive and cold-plaque screening of 200 cDNA clones from the resurrection angiosperm *C. plantagineum* leaves that had been dried, genes were isolated which had no similarity to those in the public database (Bockel et al., 1998). Almost half of those were upregulated by dehydration.

By differential screening of a dehydration cDNA library in the resurrection plant *X. viscosa*, 30 cDNA clones were found to have higher expressions in dehydrated leaf tissue than in hydrated ones (Ndima et al., 2001). An aldose reductase homologue (*ALDXV4*) was cloned from the plant and was found to confer tolerance to osmotic stress in *E. coli* cells (Garwe et al., 2003). Northern blot analysis showed that *ALDXV4* was expressed only under conditions of dehydration stress. Another gene, *XVSAP1*, isolated by using complementation by functional sufficiency, was isolated. Expression of this gene in the osmotically deficient *E. coli* strain (*srl:: Tn10*) conferred osmotic stress tolerance. This gene is highly expressed under several abiotic stresses. Over-expression in Arabidopsis and tobacco significantly improved their abilities to survive under conditions of high salinity stress, heat and osmotic stress (Garwe et al., unpublished). Several other stress-inducible genes from *X. viscosa* have been characterized and their ability to improve plants' ability to survive abiotic stress is under investigation (Mowla et al., 2002; Marais et al., 2004). It is very probable that resurrection plants possess unique gene complements or regulatory processes that contribute to desiccation tolerance.

1.6 OBJECTIVES OF THIS STUDY

The ultimate aim of this research is the development of transgenic crop plants relevant to sub-Saharan Africa, such as maize and sorghum, able to grow in traditionally unproductive soil or in conditions of environmental stress such as low rainfall and high light intensities.

This research project is divided into three distinct studies. The first involves the molecular characterization of *XvPer1*. This gene, encoding a 1-Cys peroxiredoxin antioxidant enzyme, was isolated by differential screening of a cDNA library

constructed from RNA isolated from dehydrated *X. viscosa* leaf tissue (Ndima et al., 2001).

The objectives of this study were to:

- sequence and thoroughly analyse the *XvPer1* cDNA sequence,
- confirm the presence of *XvPer1* in the *X. viscosa* genome and to investigate its copy number,
- confirm that *XvPer1* is a dehydration inducible gene and to investigate its expression under other abiotic stresses,
- investigate the expression of the XvPer1 protein under abiotic stresses,
- investigate the cellular localization of XvPer1, and
- assign a cellular function to XvPer1 using transgenic approaches.

The second study involves the molecular characterization *AC3*, a cDNA encoding a LEA-like protein isolated from the model plant *Arabidopsis* by complementation of a yeast mutant susceptible to oxidative stress. *AC3* was found to confer H₂O₂ tolerance in yeast.

The objectives of this study were to:

- analyse the cDNA sequence of *AC3*,
- investigate its expression in *Arabidopsis* under dehydration, ABA treatment and various oxidative stresses,
- investigate the antioxidant capacity of *AC3* by doing yeast complementation studies,
- investigate the signal transduction pathway of *AC3* expression,
- investigate the functions of *AC3* in *Arabidopsis* by studying *AC3* knock-out mutants, and
- further characterize *AC3* by generating and analysing *Arabidopsis* plants over- and under-expressing *AC3*.

The third study was to carry out a comparative analysis of *XvPer1* with its *Arabidopsis* orthologue *AtPer1*.

AtPer1 and XvPer1 share more than 70% identity at the protein level. However, unlike *XvPer1*, *AtPer1* is only very faintly induced by dehydration stress and is seed specific (Haslekas et al., 1998, 2003b).

The objectives of this study were to:

- analyse the cDNA sequence of *AtPer1*,
- confirm that *AtPer1* is not induced by dehydration stress and to investigate its expression under other stresses such as ABA stress and oxidative stress,
- generate and analyse Arabidopsis plants over-expressing and under-expressing the *X. viscosa* orthologue *XvPer1*, and
- determine the cellular localization of over-expressed XvPer1 in Arabidopsis by GFP fusion.

CHAPTER TWO

MOLECULAR CHARACTERIZATION OF *XvPer1*

SUMMARY

By differential screening of a cDNA library of the resurrection plant *Xerophyta viscosa* (Baker), a cDNA designated *XvPer1* corresponding to 1-Cys peroxiredoxin, an evolutionary conserved thiol-dependent antioxidant enzyme, was isolated. The analysis of the 849 bp cDNA and sequence revealed an open reading frame (ORF) encoding a hydrophilic polypeptide of 219 residues with a predicted molecular weight of 24.2 kDa. The *XvPer1* polypeptide has significant identity (approx. 70%) with other recently identified plant 1-Cys peroxiredoxins, with an absolutely conserved amino acid sequence proposed to constitute the active site of the enzyme (PVCTTE). It also contains a putative bipartite nuclear localization signal (NLS). Southern blot analysis revealed that *XvPer1* is most likely a single copy gene in the *X. viscosa* genome. In summary, *XvPer1* is likely to be a nuclear localized, stress-inducible, antioxidant enzyme, involved in the protection of nucleic acids by scavenging reactive oxygen species (ROS).

2.1 INTRODUCTION

The genes induced during dehydration stress in *X. viscosa* are thought to be responsible for the plants amazing ability to withstand and survive extreme desiccation and hence our interest in identifying, isolating and characterizing such genes. In order to study the change in gene expression in the *X. viscosa* genome from a hydrated to a dehydrated state, the strategy of differential screening of cDNA libraries was employed (Ndima et al., 2001). *X. viscosa* plants were dehydrated to 85%, 37% and 5% relative water content (RWC). Under well-watered conditions, *X. viscosa* leaf tissues are generally between 90-95% RWC. Total RNA was isolated from leaves from all three stages of dehydration. The pooled samples were used to construct a cDNA library which was differentially screened against total RNA isolated from hydrated and dehydrated *X. viscosa* leaves. Using this strategy several genes were observed to be up-regulated as well as down-regulated during dehydration. In this chapter, the isolation of *XvPer1* will be described, as well as its

molecular characterization based on the cDNA and protein sequences. Its gene copy number in the *X. viscosa* genome will also be estimated.

2.2 MATERIALS AND METHODS

2.2.1 Plant Material and Growth Conditions

Mature *Xerophyta viscosa* (Baker) plants were collected from the Buffelskloof Nature Reserve (Mpumalanga Province, South Africa) and from Cathedral Peak Nature Reserve in the Drakensberg mountains (Kwazulu-Natal, South Africa) during the October month (autumn) of 2001. The plants were grown under glasshouse conditions as described by Sherwin and Farrant (1996).

2.2.2 Isolation of *XvPer1* cDNA

By differential screening of an *X. viscosa* cDNA library, *XvPer1* was found to be one of several cDNA that were induced upon dehydration (Ndima et al., 2001). It was therefore selected for further analysis. A cDNA library was generated using a cDNA synthesis Kit (Stratagene, USA). The strategy used is outlined in Figure 2.1. The first strand cDNA synthesis involved reverse transcription with a linker-primer containing an XhoI restriction site. During second strand synthesis, RNaseH nicked the RNA template bound to the first strand cDNA to produce a number of fragments which were used as primers for DNA polymerase I. The DNA polymerase 'nick translated' these RNA fragments into second strand cDNA. The uneven ends of the cDNAs were blunted and EcoRI restriction enzyme adapters were ligated. This was then cut with the restriction enzymes XhoI and EcoRI, resulting in an EcoRI-XhoI directional cDNA. A 50 µl sample of the cDNA pool was inserted into the pBluescript phagemid and the cDNAs were cloned into the polylinker on the pBluescript vector [X52328 (SK+); X52330 (SK-)]. This plasmid system has the convenience of the blue-white selection and the directional cloning of cDNAs. The polylinker of the pBluescript has 21 unique cloning sites flanked by T3 and T7 promoters and a choice of six primer sites for DNA sequencing.

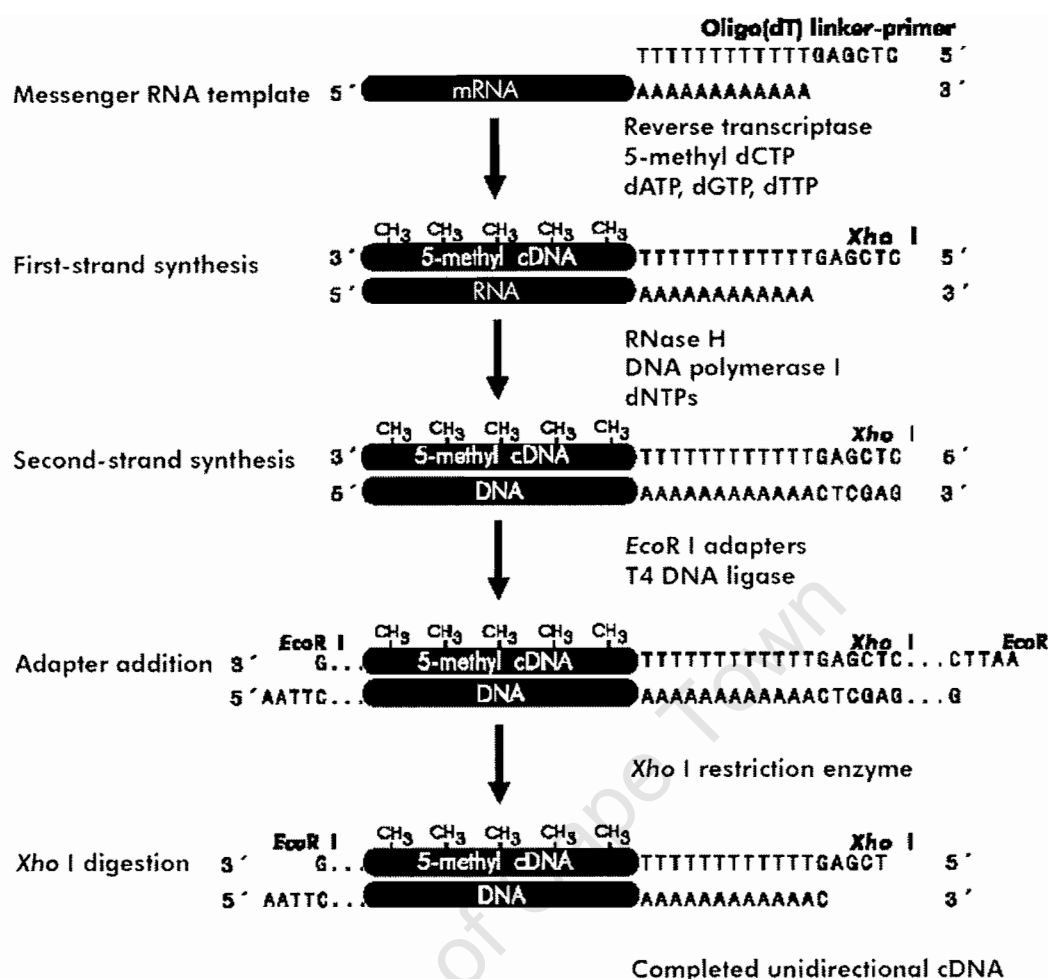


Figure 2.1. cDNA synthesis flow chart (Stratagene Bacteriomatch II Two-hybrid System Library construction Kit, USA)

The library was then differentially screened (Ndima et al., 2001). 192 cDNA clones were randomly selected from the cDNA library, and slot blotted in duplicate using standard slot blot apparatus (Hoefer Scientific, USA). Total RNA was isolated from hydrated (100% RWC) and dehydrated (37% RWC) *X. viscosa* leaves and reversely transcribed, incorporating $^{32}\text{P}[\text{dCTP}]$ (Amersham, UK). One set of the 192 cDNA clones was hybridized with labelled first strand cDNA from hydrated plants while a second set was hybridized with labelled first strand cDNA from dehydrated plants. Following autoradiography, 30 cDNA clones exhibited higher expressions under dehydrated conditions while 20 cDNA clones exhibited higher expression under hydrated conditions. A cDNA designated *XvPer1* was one of the 30 clones which were up-regulated under dehydrated conditions.

2.2.3 Sequence Analysis of *XvPer1*

The nucleotide sequence of the *XvPer1* cDNA was determined on both forward and reverse strands using the ALFexpress automated DNA sequencer AMV3.0 (Pharmacia Biotech, USA) using the sequencing Fluorescent Labelled Primer cycle sequencing kit (Amersham International). The inferred amino acid sequence of *XvPer1* was obtained by translation of the cDNA sequence using the DNAMAN software (Version 3.0, 1997). Both the cDNA and amino acid sequences were used in the BLAST programme of the National Centre for Biotechnology Information (Altschul et al., 1990; blastp; nr database) to search databases for sequence similarities. Amino acid sequence comparisons were done with the CLUSTAL programme of DNAMAN (default setting). The Bioinformatics and Biological Computing Unit, Weizmann Institute of Science, (Israel) was used to plot the hydrophilicity/hydrophobicity of *XvPer1*. The ScanProsite tool provided by ExPASy (us.expasy.org) was used to scan *XvPer1* for conserved motifs. The phylogenetic tree representing the relationship of *XvPer1* to 16 other orthologues was generated by the DNAMAN software.

2.2.4 Southern Blot Analysis of *XvPer1*

Genomic DNA was extracted from leaves of fully hydrated *X. viscosa* plants according to the procedure described by Dellaporta et al. (1983). The concentration and purity of the DNA preparations were determined spectrophotometrically. Aliquots (15 µg) were fragmented with restriction enzymes EcoRI, EcoRV and HindIII (Boehringer-Mannheim, Germany) respectively, and electrophoresed on 0.8% ethidium bromide (EtBr)-stained agarose gels. The DNA was subsequently transferred and UV cross-linked (UV Stratalinker™; Stratagene, USA) onto a nylon membrane (Hybond-XL; Amersham Pharmacia Biotech, USA), which was hybridized at 65°C with radiolabelled probe. The probe was radiolabelled by Polymerase Chain Reaction (PCR) on *XvPer1* cDNA using *XvPer1* forward primer (5'-CCATGCCGGGGCTCACCATT-3') and *XvPer1* reverse primer (5'-CATTCACTCAGACGTTCGTAAAACG-3') and using [α -³²P]dCTP. The PCR reactions were conducted using a Gene Amp 9700 (Perkin Elmer Applied Biosystems, CA, USA) thermocycler under the following conditions: 95°C for 2 minutes followed by 15 cycles of 95°C for 30 seconds, 55°C for 10 minutes and 72°C for 45 seconds

and a final extension step for 7 minutes. An unusually long extension time of 10 minutes was used to ensure that the 'heavier' radiolabelled dCTP would incorporate during amplification. Unincorporated nucleotides were removed by passing the PCR product through a sephadex-G50 column. The specific activity of the labelled probe was determined in a scintillation counter by counting 1 µl of probe in 2 µl of scintillation fluid. The membrane was prehybridized in buffer (0.5 M NaH₂PO₄, 1 mM EDTA, 7% SDS, 1% BSA) for a minimum of 2 hours. After prehybridization, the radiolabelled probe was denatured (placed in a boiling water bath for 10 minutes and then cooled on ice) and then added to the membrane. Hybridization was carried out at 60°C for 16 hours with gentle shaking. The membrane was washed twice at 60°C for 10 minutes in wash buffer B (0.1% SDS, 0.5xSSC) and autoradiographed at -70°C onto high-performance-autoradiography film (Amersham Pharmacia Biotech, USA). The above protocol was adapted from Sambrook et al. (1989). After 5 days of exposure, the film was developed manually using developer and fixer reagents (Amersham Pharmacia Biotech., UK) according to manufacturer's instructions.

2.3 RESULTS

2.3.1 Sequence Analysis of *XvPer1*

XvPer1 has a nucleotide sequence of 849 bp, representing the cDNA (Fig. 2.2). It has an open reading frame (ORF) of 657 bp with a deduced amino acid sequence encoding for a protein of 219 amino acid residues, with a molecular weight of 24.2 kDa and a predicted pI of 6.31 at pH 7. 189 bp of untranslated sequence exists after the deduced stop codon before the polyadenylation site. The genbank accession number for the gene is AF484696.

A hydropathy plot revealed that the protein is mostly hydrophilic (Fig. 2.3). A few hydrophobic domains are present, especially in proximity of residues 35 and 70 and could be indicative of the function of the protein. A scan tool suggests one possible N-myristoylation site at position 3-8, as well as five possible Casein kinase II phosphorylation sites (5-8, 71-74, 77-80, 190-193 and 209-212) and five possible Protein kinase C phosphorylation sites (61-63, 77-79, 120-122, 149-151 and 209-211).

```

1      ATGCCGGGGCTACCATTTGGCGACACGATCCCCAACCTGGAGCTTGACACCACCCAGGGT
1      M P G L T J G D T I P N L E L D T T Q G

61     AGGATCAAAATCCACGATTACGTCGGCAACGGCTACGTCATCTTGTTCTCACACCCTGGA
21     R I K I H D Y V G N G Y V I L F S H P G

121    GACTTCACTCCTGTCTGCACCACCGAACTTGGAAAGATGGCTGCTTACGCCGACGAGTTC
41     D F T P V C T T E L G K M A A Y A D E F

181    AGCAAGCGCGGGTTAAGCTTCTTGCTTTCTGCGACGATGTACAGAGCCACAAGGAG
61     S K R G V K L L G L S C D D V Q S H K E

241    TGGATCAAGGATATCGAAGCCTATACGCCGGGATGTCACGTAAAATATCCTATCGCGGCG
81     W I K D I E A Y T P G C H V K Y P I A A

301    GACCCAACCCGCGAGATTATCCAGCAGCTAAACATGGTAGACCCAGACGAGACAGAGTCC
101    D P T R E I I Q Q L N M V D P D E T E S

361    AGCAAATGCGCCGTGCCTTCGCGAGCTCTGCACATCATTGGGCCCGACAAGAGGATCAAG
121    S K C A V P S R A L H I I G P D K R I K

421    CTGAGTTTCTGTACCCCGCTCGACGGGGCGAAACATGGATGAGGTGCTGAGGGCAGTG
141    L S F L Y P A S T G R N M D E V L R A V

481    GAGTCGCTCCAGCAGGCGGCAAAGCACAAGGTGGCAACGCCGGCGAACTGGAAGCCTGGT
161    E S L Q Q A A K H K V A T P A N W K P G

541    GAACCTGTTGTGATCAAGCCTGATGTGTCCAGCGAGGAGGCCAAGAAGCTTTCCCGCAG
181    E P V V I K P D V S S E E A K K L F P Q

601    GGTATAAAAGTGTGATCTTCCATCCAAGAAGGATTACCTTCGTTTTACGAACGTCTGA
201    G Y K S V D L P S K K D Y L R F T N V *

661    GTGAATGCAAAGCCTATTACTATCTGTATCACAAGAGTAGTTTTGGTGTAAATCTTCT
721    GCTACTATGATCTACTACGGTGTTCCTTTTGGTTATGTATTTCTTGAGGCGATGAAAGC
781    TTTATGCTGCTGTATTATCCAAATATGTGCCCTACATATATTATACTTAATCATATATAT
841    TAACAAAAA

```

Figure 2.2. Coding nucleotide region and deduced amino acid sequence of *XvPer1*. The cDNA is 849 bp long and has two putative polyadenylation sites at the 3-end (underlined). The amino acid sequence has an ORF from 1 to 657, 219 amino acids and a molecular weight of 24223 Da. The N-myristoylation site is indicated by broken lines (3-6). The five Casein kinase II phosphorylation sites are indicated as red amino acids and the five Protein kinase C phosphorylation sites are indicated as amino acids in italics. The highly conserved active site PVCTTE is boxed at position 44-49 and the putative bipartite NLS is highlighted at positions 195, 196; 210-211; 214-217. The stop codon is indicated by * (Genbank accession number: AF484696) (adapted from Mowla et al., 2002).

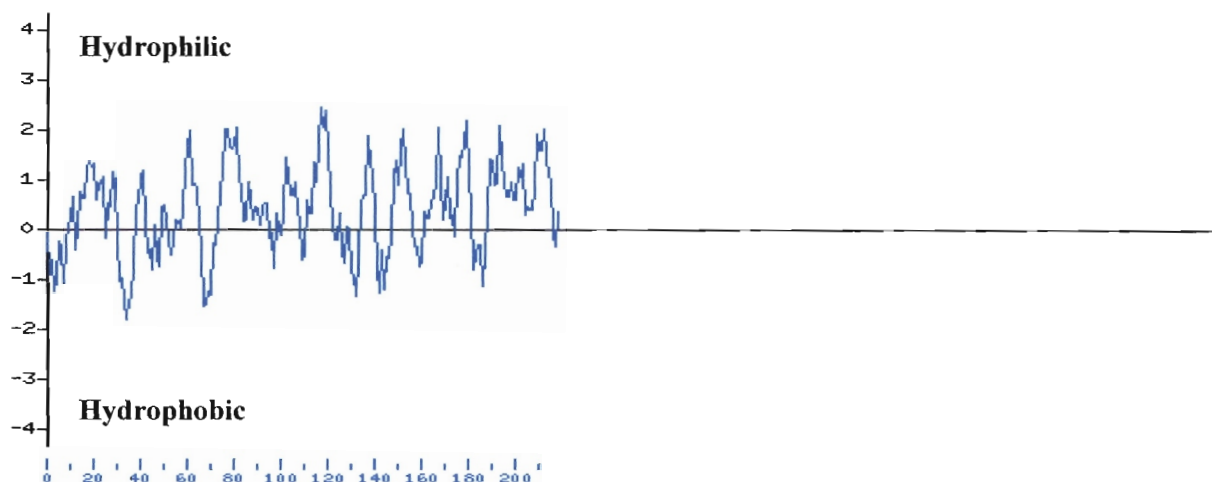


Figure 2.3. A hydropathy profile of the XvPer1 protein as determined by the method of Kyte and Doolittle (1982) using the Bioinformatics and Biological Computing Unit, Weizmann Institute of Science (Israel).

XvPer1 contains the highly conserved PCVTTE amino acid sequence, in which the cysteine residue constitutes the active site of the enzyme and is characteristic of all thiol-specific 1-Cys peroxiredoxin proteins isolated thus far (Fig. 2.2). A putative bipartite nuclear localization signal (NLS) is also present, which is conserved in all plant 1-Cys Prx proteins (Raikhel, 1992) although a topology search revealed that XvPer1 is most likely to be cytoplasmic. Two hexanucleotide sequences, ATATAT, found at the 3'-end of the sequence, represent a putative polyadenylation signal of this gene (Wu et al., 1995). The deduced amino acid sequence of XvPer1 exhibited considerable similarity to other plant 1-Cys Prx orthologues (Fig. 2.4). XvPer1 shares 77% similarity with the *Bromus secalinus* orthologue (Goldmark et al., 1992; p52572), 77% similarity with the *Hordeum vulgare* orthologue (Aalen et al., 1994; p52572), 74% similarity with the *Oryza sativa* orthologue (Fujino et al., unpublished; p52573), 72% similarity with the *Arabidopsis* orthologue (Haslekas et al., 1998; 0040005) and 72% similarity with the *Fagopyrum esculentum* orthologue (Lewis et al., 2000; AAF12782).

XvPer1	MPGLTIGDTI	PNLELDTTQG	RIKIHDYVGN	GYVILFSPHG	DFTPVCTTEL
<i>B. secalinus</i>STHG	KIRIHDYVAN	GYVILFSPHG	DFTPVCTTEL
<i>H. vulgare</i>	MPGLTIGDTV	PNLELDSTHG	KIRIHDYVGN	GYVILFSPHG	DFTPVCTTEL
<i>O. sativa</i>	MPGLTIGDTV	PNLELDSTHG	KIRIHDFVGD	TYVILFSPHG	DFTPVCTTEL
<i>A. thaliana</i>	MPGITLGDTV	PNLEVETTHD	KFKLHDYFAN	SWTVLFSPHG	DFTPVCTTEL
<i>F. esculentum</i>	MPGLTIGDSI	PNLQVETTHG	SFKLHDFIGD	SWVILFSPHG	DFTPVCTTEL
		..*.**....*****	*****
XvPer1	GKMAAYADEF	SKRGVKLLGL	SCDDVQSHKE	WIKDIEAYTP	GCHVKYPIAA
<i>B. secalinus</i>	AAMANYAKEF	EKRGVKLLGI	SCDDVQSHKE	WTKDIEAYKP	GSKVITYPIMA
<i>H. vulgare</i>	AAMANYAKEF	EKRGVKLLGI	SCDDVQSHKE	WTKDIEAYKP	GSKVITYPIMA
<i>O. sativa</i>	AAMAAAYAKEF	DKRGVKLLGI	SCDDVQSHKD	WFKDIEAYKP	GNRVITYPIMA
<i>A. thaliana</i>	GAMAKYAHEF	DKRGVKLLGL	SCDDVQSHKD	WIKDIEAFNH	GSKVNYPIIA
<i>F. esculentum</i>	GKMAKYEEEF	TKRGVKLLGL	SCDDIASHKE	WIKDVEAFTP	GSKVRYPIIA
	..**.*..**	*****	****.*	*..**.*..	*..**.*..
XvPer1	DPTREIIQQL	NMVDPDETES	SKCAVPSRAL	HIIGPDKRIK	LSFLYPASTG
<i>B. secalinus</i>	DPDRSAIKQL	NMVDPDEKDA	EGQ.LPSRTL	HIVGPDKKVK	LSFLYPSCCTG
<i>H. vulgare</i>	DPDRSAIKQL	NMVDPDEKDA	QGQ.LPSRTL	HIVGPDKVVK	LSFLYPSCCTG
<i>O. sativa</i>	DPSREAIKQL	NMVDPDEKDS	NGGHLPSRAL	HIVGPDKKVK	LSFLYPACVG
<i>A. thaliana</i>	DPNKEIIPQL	NMIDPIENG.PSRAL	HIVGPDSEKIK	LSFLYPSTTG
<i>F. esculentum</i>	DPKREVITKL	NMVDPDEKDS	SGSQLPSRAL	HIVGPDKKVK	LSFLYPATTG
*	**.*	*.*	**.*	*****
XvPer1	RNMDEVLRV	ESLQQAQKH	V..ATPANWK	PGEFVVIKPD	VSSEEAKKLF
<i>B. secalinus</i>	RNMDEVVRV	DSLTLAAKH	V..ATPANWK	PGECVVIAPG	VSDEEAKKLF
<i>H. vulgare</i>	RNMDEVVRV	DSLTLAAKH	V..ATPANWK	PGECVVIAPG	VSDEEAKKMF
<i>O. sativa</i>	RNMDEVVRV	DALQTAAKTR	L..ATPVNWK	PGEFVVIKPD	VSDDEAKEKF
<i>A. thaliana</i>	RNMDEVLRV	DSLIMASKHN	NKIATPVNWK	PDQPVVISPA	VSDEEAKKMF
<i>F. esculentum</i>	RNMDEVVRV	ESLQQAANDK	V..ATPVDWQ	PGDEAVISPS	VSDEEAKKMF
	.*	***.*	**	*****
XvPer1	PQGYKSVDLP	SKKGYLRFTN	V.	100%	
<i>B. secalinus</i>	PQGFETKDL	SKKGYLRFTK	V.	77%	
<i>H. vulgare</i>	PQGFETADLP	SKKGYLRFTK	V.	77%	
<i>O. sativa</i>	PQGFDTADLP	SGKGYLRFTK	VG	74%	
<i>A. thaliana</i>	PQGFKTADLP	SKKGYLRHTE	VS	72%	
<i>F. esculentum</i>	PHGYRTVDLP	SKKGYLRFTQ	V.	72%	
	*..**.*	*..**.*	*		

Figure 2.4. Multiple sequence alignment of 1Cys-peroxiredoxin orthologous sequences from *Bromus secalinus* (p52571), *Hordeum vulgare* (p52572), *Oryza sativa* (p52573), *Arabidopsis thaliana* (0040005) and *Fagopyrum esculentum* (AAF12782) with XvPer1 (in blue). Asterisks (*) denote identities and dots (.) denote similarities. The highly conserved PVCTTE domains of the active sites of the enzymes are highlighted in grey (adapted from Mowla et al., 2002).

Over 50 members of the peroxiredoxin family have been identified in organisms from all kingdoms. In an attempt to determine the evolutionary relationships between 1-Cys peroxiredoxin family members, a phylogenetic tree was generated which revealed that XvPer1 is most closely related to its rice orthologue (Fig. 2.5)

might be due to contamination of genomic DNA with chloroplastic DNA. The choice of enzymes was to reveal the presence or absence of an intron in the *XvPer1* fragment. The Southern blot analysis was carried out to confirm the presence of *XvPer1* in the *X. viscosa* genome as well as to estimate its gene copy number. An exact copy number cannot be determined without using the full-length *XvPer1* genomic sequence as probe. However, using the full-length cDNA sequence instead does shed some light on the amount of copies present in the genome.

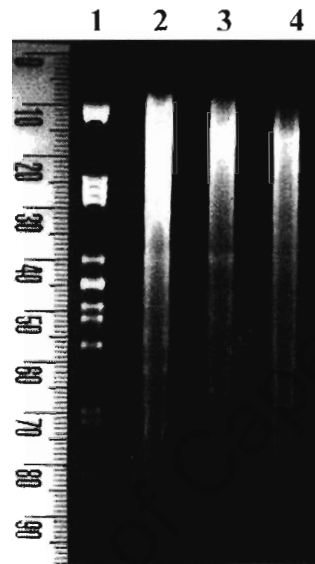


Figure 2.6. Ethidium bromide (EtBr)-stained 0.8% agarose gel electrophoresis of *X. viscosa* genomic DNA used for Southern analysis of *XvPer1*. 1- λ -PstI marker; 2- genomic DNA digested with EcoRI; 3- genomic DNA digested with EcoRV. 4- genomic DNA digested with HindIII

The Southern blot analysis of total genomic DNA from hydrated (100% RWC) *X. viscosa* leaves revealed that *XvPer1* was most probably a single copy gene (Fig. 2.7). The restriction enzymes EcoRI and EcoRV do not cut inside the cDNA transcript and gave single bands of 9 kb and 14 kb respectively. HindIII cuts the cDNA transcript twice and hence three bands were observed (0.9 kb, 0.4 kb and 0.2 kb), with the 0.4 kb band corresponding to the internal *XvPer1* fragment. Fainter bands were also observed in all three lanes which might be due to non-specific hybridization (low stringency wash) or caused by partial digestion of the genomic DNA.

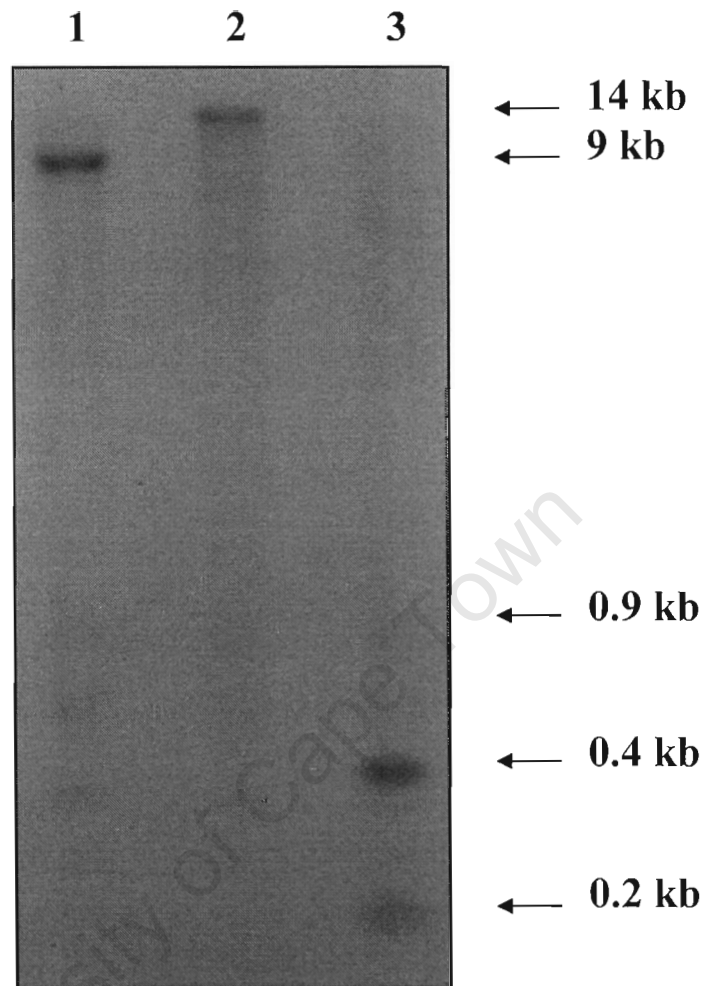


Figure 2.7. Southern blot analysis of *XvPer1*. Twenty micrograms (20 µg) of genomic DNA isolated from hydrated leaf tissue of *X. viscosa* was digested with EcoRI (lane 1), EcoRI (lane 2) and HindIII (lane 3), fractionated by electrophoresis, transferred onto nylon membrane and hybridized with ³²P-labelled *XvPer1* cDNA probe. The molecular weights of the bands observed are indicated in the right margin (adapted from Mowla et al., 2002).

2.4 DISCUSSION

By screening of a cDNA library from *X. viscosa*, a stress-inducible gene named *XvPer1* was isolated. The data presented in this study indicates that *XvPer1* encodes a 1-Cys peroxiredoxin, which is a newly discovered class of thiol-dependent antioxidant enzyme (Mowla et al., 2002). The cDNA sequence is 849 bp in length, containing one major open reading frame of 657 bp (Fig. 2.2). Two putative

polyadenylation signals, ATATAT, were identified 155 bp and 172 bp downstream of the stop codon. The amino acid sequence deduced from the cDNA indicates that *XvPer1* encodes a polypeptide of 219 amino acid residues with a predicted molecular mass of 24.2 kDa and a predicted pI of 6.31 at pH 7. The relatively small size of the protein coincides with other 1-Cys Prx proteins characterized in both plants and mammals (Haslekas et al., 1998; Kang et al., 1998; Stacy et al., 1999). A hydropathy plot of the deduced amino acid sequence of XvPer1 revealed no major hydrophobic domains and two minor hydrophobic domains, which indicates that XvPer1 is a mostly hydrophilic protein and unlikely to be associated with membranes (Fig. 2.3). One possible N-myristoylation site exists in the protein as well as five possible Casein kinase II phosphorylation sites and five possible Protein kinase C phosphorylation sites. Protein N-myristoylation refers to the covalent attachment of myristic acid by an amide bond to the N-terminal glycine residue of a nascent polypeptide (Johnson et al., 1994). This co-translational modification occurs on many proteins involved in signal transduction, including serine/threonine kinases, tyrosine kinases, calcium binding proteins etc. In most cases, this modification is essential for protein function to mediate membrane association or protein-protein interaction. In some cases however, no functional significance can be associated with myristoylation. Protein phosphorylation is probably the most important regulatory event and many enzymes are switched 'on' or 'off' by phosphorylation and dephosphorylation. The presence of these phosphorylation sites could be an indication of the type of regulation of the XvPer1 protein.

XvPer1 has more than 70% identity with other plant 1-Cys Prx proteins (Fig. 2.4) (Aalen et al., 1994; Fujino et al., unpublished; Haslekas et al., 1998; Lewis et al., 2000; Mowla et al., 2002). Peroxiredoxins represent a highly conserved family of proteins found in organisms as widely divergent as the *Archea* bacteria, plants and humans (Fig. 2.5) (Kang et al., 1998). The conservation of the amino acid sequence among 1-Cys Prx proteins identified from widely divergent species indicates its importance in function. XvPer1 contains the highly conserved PVCTTE amino acid sequence (44-49) where the cysteine residue at position 46 constitutes the active site of the enzyme and is conserved in all 1-Cys Prx proteins isolated. For the human 1-Cys Prx, site-directed mutagenesis studies have demonstrated that this conserved

cysteine residue is essential for the function of the protein, constituting the site of oxidation (Kang et al., 1998).

Stacy et al. (1999) showed that the dormancy-related barley peroxiredoxin orthologue, PER1, is localized in the nucleus of immature embryo and aleurone cells. In XvPer1, a putative bipartite NLS was identified in the carboxyl-terminal and of the protein sequence, which is conserved in all plant 1-Cys Prx proteins (Lewis et al., 2000). This would suggest that this enzyme is targeted to the nucleus. However, a similar core sequence is also present in the human 1-Cys Prx orthologue, which has been localized to the cytosol (Kang et al., 1998; Stacy et al., 1999). The localization of XvPer1 has been investigated and is presented at a later stage in this study.

XvPer1 appears to be encoded by a single copy gene. This is characteristic of all plant 1-Cys Prx proteins isolated (Stacy et al., 1996; Haslekas et al., 1998). The first plant 1-Cys Prx was isolated from the seeds of *B. secalinus*, a grass species, while the authors were investigating the mechanisms involved in seed dormancy (Goldmark et al., 1992). The next one identified, B15C (later re-named PER1), was discovered in barley (*H. vulgare* L.) seeds (Aalen et al., 1994). Thereafter, a 1-Cys Prx gene, *AtPer1*, was isolated from the seeds of the dicotyledonous plant *Arabidopsis* (Haslekas et al., 1998). In all of the above, transcripts of the gene disappeared after germination and were absent or present in barely detectable quantities in vegetative tissues even under dehydration stress (Halekas et al., 2003b). The transcript was always specific to the aleurone layer and the embryo of the seeds. The authors postulated that the protein performs a protective function in the only two tissues that survive desiccation. A second role postulated was its involvement in the maintenance of seed dormancy.

X. viscosa is unique in that it is the first angiosperm in which abundant levels of a 1-Cys peroxiredoxin has been found in vegetative tissues. The orthologue from the resurrection moss *T. ruralis* (bryophyte), Tr155, is expressed in the gametophyte in response to desiccation and rehydration (Wood et al., 1999). However, no published data has been found in relation to its activity or localization. Rab24, the rice orthologue, was found in callous tissue (reported in GenBank entry), but there is no published evidence of expression in the vegetative tissues of *O. sativa* plants. An

examination of the sequence of XvPer1 has revealed that it might function as an anti-oxidant enzyme. Its putative localization to the nucleus suggests a role in the protection of nucleic acids against damage by ROS.

University of Cape Town

CHAPTER THREE

***XvPer1* cDNA AND PROTEIN EXPRESSIONS UNDER ABIOTIC STRESSES**

SUMMARY

The *XvPer1* cDNA was isolated by differential screening of an *X. viscosa* cDNA library constructed from RNA isolated from dehydrating vegetative tissues. It was therefore concluded that *XvPer1* was induced in response to water deficit. Northern blot analysis was used to confirm that *XvPer1* was indeed induced by dehydration in *X. viscosa*. The *XvPer1* steady-state mRNA transcript was absent in fully hydrated leaf tissue but levels increased in tissues subjected to dehydration. Its expression under several other artificially imposed stresses was also investigated. *XvPer1* cDNA transcript levels increased under heat (42°C), high light intensity (1500 $\mu\text{molm}^{-2}\text{s}^{-1}$), and when treated with abscisic acid (100 μM ABA) and sodium chloride (100 mM NaCl). To investigate the expression of the *XvPer1* protein under similar stresses, an *XvPer1* antibody was generated from purified recombinant *XvPer1* protein produced in a prokaryotic system. Western blot analyses correlated with the patterns of expression of *XvPer1* transcripts under the different stress conditions except for high light stress. These results therefore suggest that *XvPer1* is a stress-inducible gene and have an important role to play in response to water deficit stress.

3.1 INTRODUCTION

XvPer1 was isolated because of its over-expression under conditions of dehydration stress in *X. viscosa* leaves. Previous studies have shown that such genes are also often responsive to other environmental stresses such as cold and high salinity (Shinozaki & Yamaguchi-Shinozaki, 1997). Water stress induces various biochemical and physiological responses in plants, and occurs not only under drought and high salt concentrations, but also under low temperature conditions. As a result, plant cells lose water and turgor pressure inside the cell decreases. The level of the plant stress hormone abscisic acid (ABA) increases as a result of water stress and is an important signalling molecule during stress response. Quite often several different abiotic stresses share the same signalling pathways leading to the expression of the stress-

inducible genes which are thought to have a role in protecting the plant cell against water deficit (Bray, 1997). The expression patterns of stress-inducible genes are complex. Some genes respond very rapidly, whereas others are induced slowly after ABA accumulation (Shinozaki & Yamaguchi-Shinozaki, 1997). However, some genes are not responsive to exogenous ABA treatment, which suggests that they are regulated by an ABA-independent signal transduction pathway.

In this chapter, by northern blot analysis, the steady-state mRNA expression of *XvPer1* will be investigated under artificially imposed stress conditions such as dehydration/rehydration, heat, salinity, cold, ABA as well as high light intensity. High light can be very damaging to plants as it is frequently associated with damage to the photosynthetic reaction centre (Strid et al., 1994). Photosystem II is a major target for photoinhibition of photosynthesis, which occurs when the amount of light available exceeds that necessary for photosynthetic processes. Consequently, there is excessive production of ROS, leading to oxidative damage. Since *XvPer1* is thought to be involved in the scavenging of ROS, it is expected to be induced under high light stress. However the formation of ROS by photo-oxidation is not only linked to high light stress but also to other environmental stresses including water deficit stress (Sherwin & Farrant, 1998; Loggini et al., 1999), air pollutants (Van Camp et al., 1994), UV radiation (Dai et al., 1997), herbicides (Jung et al., 2000), and extreme temperatures (Jagtap and Bhargava, 1995).

Investigation of the expression of the steady-state mRNA of *XvPer1* is however not a definite indication that the protein is expressed as well. Some of the regulation of the synthesis of proteins occurs through regulation of transcription of the mRNAs encoding those proteins. There is no guarantee that the presence of the mRNA transcript of a gene is also an indication that the transcript is being translated into a protein. Although presence of protein does not guarantee function, it is a better indication activity than transcript detection alone.

In this chapter, the expression of the *XvPer1* steady-state mRNA expression under various artificially imposed stress conditions in *X. viscosa* will be described and analysed. The expression of purified *XvPer1* protein and production of antibodies will be described as well. Additionally, the expression of *XvPer1* protein will be

investigated under those stresses which gave interesting results in the northern analysis.

3.2 MATERIALS AND METHODS

3.2.1 Plant Material and Growth Conditions

X. viscosa plants were collected and cultivated as described in section 2.2.1

3.2.2 Plant Stress Treatments

All plant treatments were performed in a phytotron at 25°C, 50% humidity, a photon flux density (PFD) of 300 $\mu\text{molm}^{-2}\text{s}^{-1}$, and a day/night cycle of 16/8 hours, unless otherwise stated. Dehydration/rehydration, heat, high light and cold treatments were done on whole plants while NaCl and ABA treatments were done on excised leaves from healthy plants. Leaf samples were taken by cutting off leaves (3-5 leaves; mid-age; middle portion), immediately wrapping in aluminium foil, flash-freezing in liquid nitrogen and subsequently storing at -70°C until RNA and protein isolation. Each treatment was done in triplicate. The relative water contents (RWC) and water potentials (ψ_w) were determined for each treatment.

Dehydration

Dehydration stress was carried out by withholding water from the soil over a period of 25 days, at the end of which the leaf RWC was ~4%. Leaves were sampled at ~78%, ~63%, ~51%, ~44% and ~4% RWC. Rehydration took place when the plants were re-watered and returned to full turgor 5 days after watering. Samples of rehydrating leaves were taken at ~4%, ~32%, ~42% and ~92% RWC. The samples from this dehydration/rehydration experiment were used for the northern blot analysis. For the western blot analysis, dehydrating leaf samples were taken at ~95%, ~74%, ~51%, ~36%, ~11%, ~7% and ~6% RWC; and rehydrating leaf samples were taken at ~35%, ~80% and ~95% RWC.

Heat Stress

Heat stress was imposed by increasing the temperature in the phytotron to 42°C, leaving all other parameters constant. The plants were watered everyday to eliminate

the chance of dehydration stress. Sampling was at 0, 6, 12, 24, 48, 72 and 96 hours from start of the treatment.

Cold Stress

Cold stress was carried out in a chamber kept at 4°C with a photon flux density (PFD) of approximately 250 $\mu\text{molm}^{-2}\text{s}^{-1}$ and a 16/8 hours day/night cycle. The plants were kept well hydrated. The experiment was carried out over a period of 7 days with sampling at 24 hour intervals.

High light Stress

High light stress was imposed by placing the plants close to a high light source in the phytotron. The PFD was approximately 1500 $\mu\text{molm}^{-2}\text{s}^{-1}$, while temperature and humidity were kept constant. The experiment was carried out over 4 days with sampling at 24 hour intervals.

Salinity stress and ABA treatment

The salinity stress and ABA treatment were imposed on leaves excised from whole plants and the cut ends were submerged in either 100 mM NaCl or 100 μM ABA solution. Excised leaves were used to ensure a quicker response than when the plants are watered with the solutions of NaCl or ABA. At specific time points the leaves were removed and flash-frozen. A wounding control was performed as well using sterile distilled water. For the NaCl treatment, sampling times were at 24 hour intervals over a period of 7 days for the northern analysis, and at 0, 6, 12, 24, 48, 72 and 96 hours after start of treatment for the western analysis. For the ABA treatment, sampling times were 0, 6, 12, 24, 48 and 72 hours after start of treatment.

Relative Water Content (RWC) and Water Potential (ψ_w) measurements

The RWC was determined at each sampling point for each treatment. The initial weight (W_i) of each sample was taken before immersing it in sterile distilled water. The weight at full turgor (W_t) was taken and leaf samples were then dried at 95°C for 24 hours and the dry weight was recorded (W_d). The formula previously determined by Jin et al., (2000) was used to calculate the RWC: $\text{RWC} = [(W_i - W_d) / W_d] / [(W_t - W_d) / W_d] \times 100$.

The water potential (ψ_w) was also determined at each sampling point using a thermocouple psychrometer (Aqualab 1.5; decagon, Washington, USA) following the manufacturer's instructions. Average RWC and (ψ_w) values (determined in triplicate) for each treatment were plotted on separate axes using the Microsoft Excel package (Microsoft Office 2000).

3.2.3 Total RNA and Soluble Protein Isolation

Total RNA was extracted from control and stress-treated *X. viscosa* leaves using the Trizol reagent and following the manufacturer's instruction (Life Technologies, Germany). All solutions used, where appropriate, were treated with 0.01% diethylpyruvate (DEPC) (Sigma, UK) and all equipments used for isolation were autoclaved twice. *X. viscosa* leaves (~200 mg) were ground in liquid nitrogen and homogenized in 0.75 ml of Trizol reagent. After incubation for 5 minutes at room temperature (RT), 0.2 ml chloroform was added and samples shaken for 25 seconds followed by a further incubation at RT for 10 minutes. Samples were centrifuged at 12000 rpm for 10 minutes at 4°C and the RNA in the aqueous phase was precipitated with isopropanol. The total RNA was quantified spectrophotometrically, separated on a 1.2% agarose gel stained with EtBr to check the integrity.

Soluble protein was isolated using Trizol reagent and a modified protocol. After the aqueous phase has been removed for RNA precipitation, 0.3 ml of absolute ethanol was added to the remaining phases, the tubes were inverted and incubated at RT for 2 minutes. Thereafter the samples were centrifuged at 3000 rpm for 7 minutes. The supernatant was transferred into a new tube. This represents the soluble proteins. 1.5 ml of isopropanol was added and incubated at RT for 10 minutes, followed by centrifugation at 12000 rpm for 10 minutes at 4°C. The pellets were washed thrice with 2 ml of 0.1M ammonium acetate in 100% methanol and then washed once with 2 ml cold acetone. The air-dried pellets were resuspended in Laemmli Buffer (2.5 mM Tris, 0.2 M Glycine, 0.1% SDS).

3.2.4 Northern Blot Analysis

10 µg of RNA samples were electrophoresed in a 1.2% EtBr-stained agarose gel, and then transferred and UV cross-linked onto a nylon membrane (Hybond-XL; Amersham Pharmacia Biotech). To estimate that equal amounts were loaded, the RNA was visualised by EtBr-staining. Prehybridization (minimum of 2 hours) and hybridization were carried out at 65°C in buffer used for Southern hybridization (section 2.2.4). The probe used was prepared as described in section 2.2.4. The membranes were washed in buffer B (section 2.2.4) for 10 minutes at 65°C and exposed to high-performance film (Amersham Pharmacia Biotech) at -70°C and thereafter developed after 72 hours of exposure.

3.2.5 Cloning of *XvPer1* in a Prokaryotic Expression vector

The pProEXTM HT prokaryotic expression system (Life technologies, USA) was used to express recombinant XvPer1 protein in *E. coli*. The protein was expressed fused to a 6-histidine sequence (His)₆ which enables affinity purification. The *XvPer1* cDNA was excised from the pGem-T-Easy vector (Promega, USA) and sub-cloned directionally between the BamHI and NotI restriction enzyme sites of the multiple cloning site (MCS) of pProEXTM HTa (Fig. 3.1). The pProEXTM HTa vector contains a 6x histidine affinity tag for ease of purification of the expressed protein, a 7-amino acid spacer arm, the TEV protease recognition site for cleavage of the 6x histidine from the protein, and an extensive MCS. The *trc* promoter and *lacI^f* gene enable inducible expression of the cloned gene with isopropyl-β-D-thiogalactoside (IPTG). The pProEXTM HTa-XvPer1 plasmid construct was sequenced using the M13/pUC reverse primer (5'-AGCGGATAACAATTTTCACACAGG-3') to ensure that the sequence was cloned in frame with the histidine tag and without any mutations. *E. coli* competent cells (DH5α) were transformed with the pProEXTM HTa-XvPer1 construct and successful transformants were selected on ampicillin (100 µg/ml).

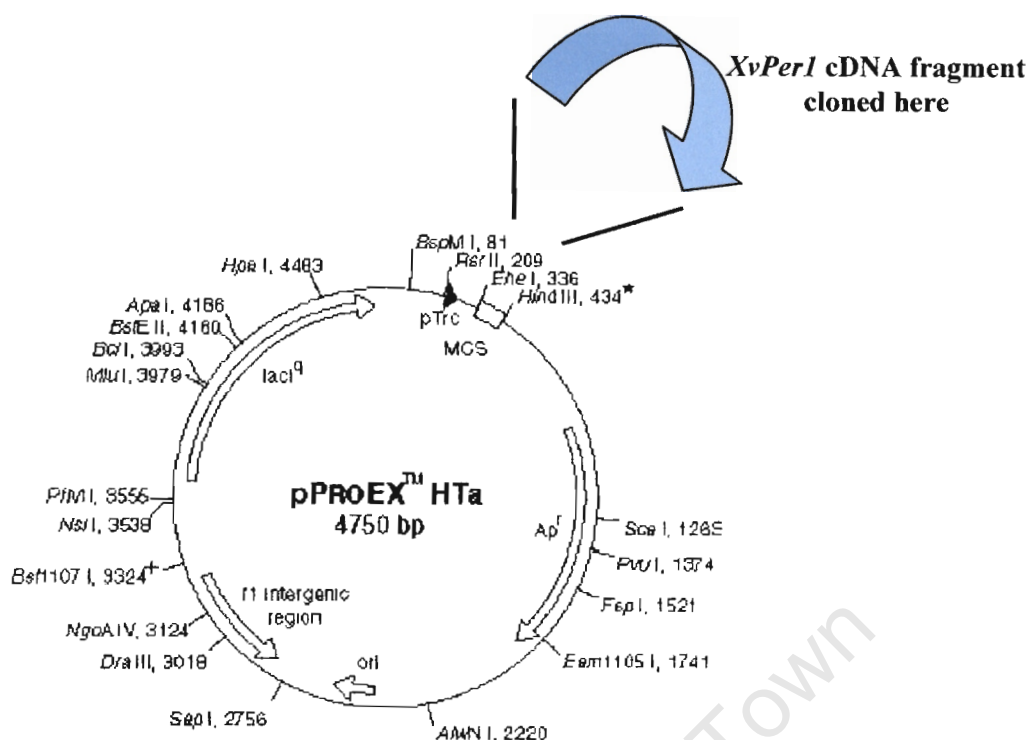


Figure 3.1. Map of pProEXTM HTa expression vector. The MCS starts with the EhaI restriction site and ends with the HindIII restriction site. The curved arrow indicates the region and the orientation of cloning of the *XvPer1* full-length cDNA fragment.

3.2.6 Expression and Purification of the Recombinant XvPer1 Protein

A single colony (*E. coli* containing pProEXTM HTa-XvPer1) of the transformed plate was used to inoculate 5 ml of culture containing antibiotic (100 µg/ml ampicillin) and incubated overnight (O/N) at 37°C. The O/N culture was used to inoculate a bigger culture of 50 ml. The optical density of the culture at A₅₉₀ was monitored until it reached 0.6-1.0. At this point, an aliquot of the culture was removed as an uninduced sample. To the rest of the culture IPTG was added to a final concentration of 0.6 mM. The culture was moved from the 37°C incubator to the 30°C incubator to optimize protein expression. Samples were collected hourly, up to 6 hours after induction, and O/N. Expression of recombinant XvPer1 protein was verified by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE). 1 ml of each collected cell culture was pelleted at maximum speed for 1 minute. The pellets were resuspended in 100 µl of PAGE disruption mix (125 mM Tris-HCL pH 7.6, 10% β-mercaptoethanol, 10% SDS, 10% sucrose, bromophenol blue) and the samples were frozen at -20°C O/N. After thawing the samples were vortexed for 5 minutes and boiled for 10 minutes followed by another 5 minutes of vortexing. This was to ensure

complete disruption of the cells for release of all soluble proteins. 10 μ l of the uninduced, the 4 hour sample and the O/N sample were separated on a 10% SDS-PAGE gel followed by coomassie staining (method obtained from Sambrook et al., 1989). To purify the recombinant XvPer1 protein, the QIAexpressTM expression system (Qiagen, Germany) was chosen. This system is based on the selectivity and affinity of nickel-nitriloacetic acid (Ni-NTA) metal-affinity chromatography matrices for biomolecules which have been tagged with six consecutive histidine residues (Qiagen The QIAexpressionist, 1997). Illustrated in Figure 3.2 is the interaction that takes place between Ni-NTA and a 6xHis-tagged protein.

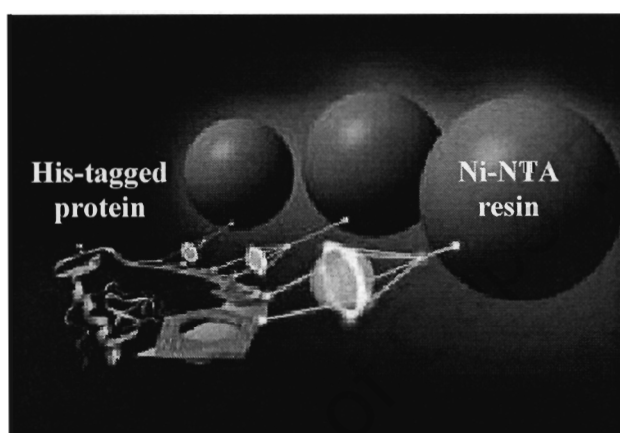


Figure 3.2. Interaction between Ni-NTA and 6xHis-tagged protein (The QIAexpressionist, Qiagen; 1997)

Nitrilotriacetic acid (NTA) is a tetradentate chelating adsorbant. NTA binds metal ions far more than other available chelating resins, and retains the ions under a wide variety of conditions, especially under stringent washing conditions (Hochuli, 1989 cited in The QIAexpressionist, Qiagen, 1997). This system allows for expression, purification, detection, and assay of 6xHis-tagged proteins. The one-step purification can be carried out under native or denaturing conditions and the pure protein products are ready for direct use in downstream applications (Fig. 3.3). Moreover, the tag does not interfere with the structure and function of the recombinant protein. No removal of the tag is necessary prior to the use of the recombinant protein as an antigen for antibody production. XvPer1 recombinant protein required for the production of antibodies was purified under denaturing conditions. Under such conditions, the 6XHis-tag of the protein is fully exposed such that binding to the Ni-NTA matrix is improved

and the efficiency of the purification procedure is maximised by reducing non-specific binding (The Qiaexpressionist, Qiagen; 1997).

A 5 ml O/N culture of *E. coli* cells containing the pProEXTM HTa-XvPer1 construct was used to inoculate a bigger (500 ml) culture. After the optical density of the culture at A₅₉₀ had reached 0.6, IPTG was added to induced recombinant protein production. 3 hours after induction, the cells were pelleted by centrifugation at 6000xg for 10 minutes. The supernatant was discarded and the pellet was stored at -70°C O/N. The rest of the procedure was carried out at 4°C. The pellet was resuspended in 4 volumes of lysis buffer (50 mM Tris-HCl pH 8.5, 100 mM KCl, 5 mM β-mercaptoethanol, 1 mM PMSF). 80 μl of lysozyme (10 mg/ml stock) was added per gram cells and the tubes left on ice for 20 minutes. Triton X-100 was added to 1% and the tubes placed at 37°C for 15 minutes. 5 μl of DNaseI (1 mg/ml stock) and 5 μl of RNaseA (10 mg/ml) were added per ml of lysate and placed at 30°C until no longer viscous. The tubes were centrifuged at 10000xg for 15 minutes. The pellet and supernatant were separated. The pellet was resuspended in lysis buffer. A 5 μl aliquot of the solubilized pellet and of the supernatant were run on an SDS-PAGE gel to confirm the location of the recombinant protein in the supernatant. To isolate the protein, the supernatant fluid was applied (flow rate = 0.5 ml/minute) to a 2 ml Ni-NTA resin column equilibrated with buffer A [20 mM Tris-HCl (pH 8.5 at 4°C), 100 mM KCl, 20 mM imidazole, 10 mM β-mercaptoethanol, 10% glycerol] (Polayes and Hughes; 1994). The column was washed with ten volumes of buffer A, followed by 2 volumes of buffer B [20 mM Tris-HCl (pH 8.5 at 4°C), 1 M KCl, 10 mM β-mercaptoethanol, 10% glycerol], and then re-equilibrated with 2 volumes of buffer A. The His-tagged protein was eluted as 1 ml fractions with buffer C [20 mM Tris-HCl (pH 8.5 at 4°C), 100 mM KCl, 100 mM imidazole, 10 mM β-mercaptoethanol, 10% glycerol). Protein fractions were analyzed by SDS-PAGE and the protein was quantified by the method of Bradford (Bradford; 1976).

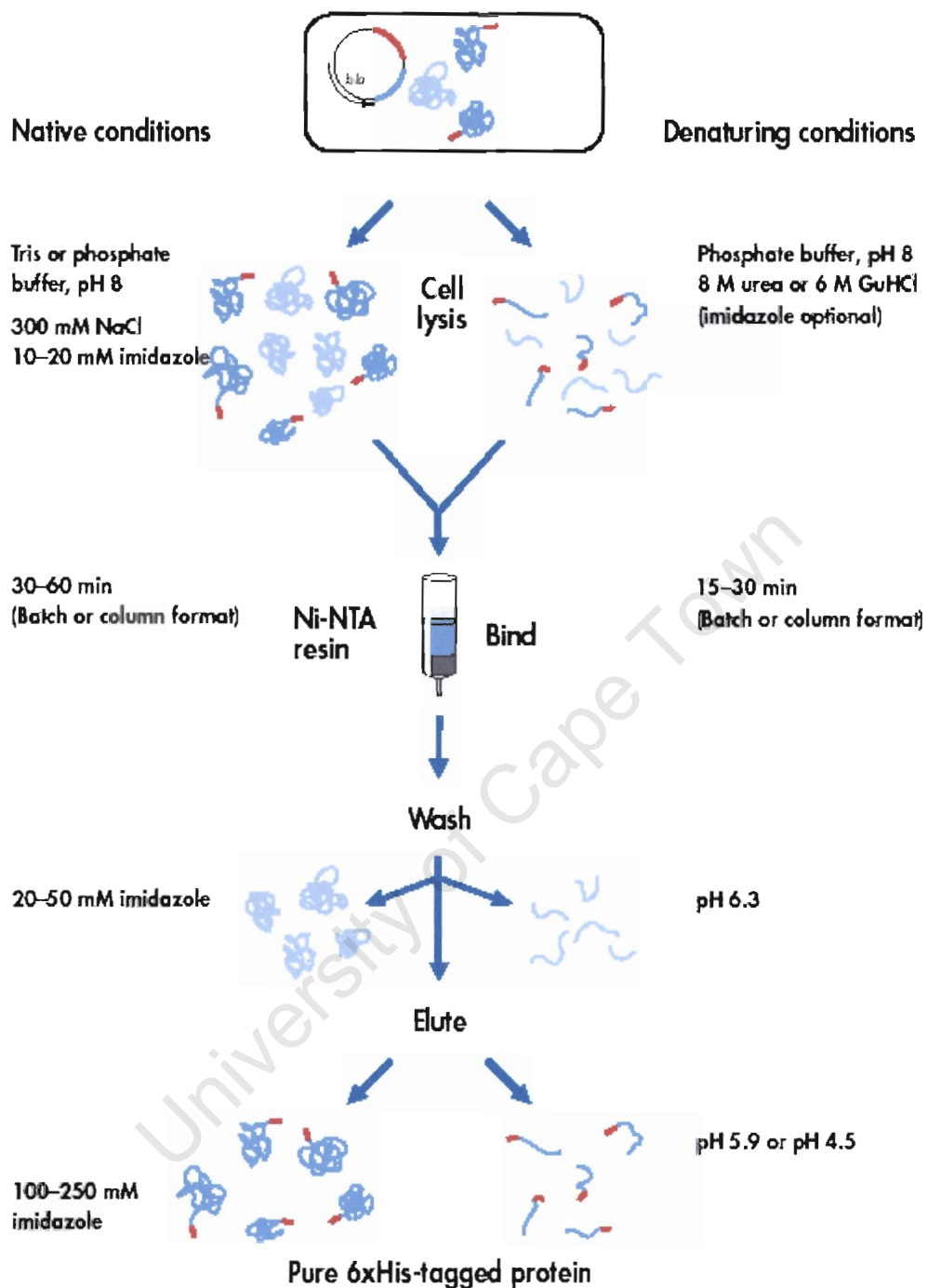


Figure 3.3. Purification of 6XHis-tagged proteins using the QIAexpress system (The Qiaexpressionist, Qiagen; 1997).

3.2.7 Production of Antibodies against XvPer1 and Purification by IgG isolation

Polyclonal XvPer1 antibody production was achieved by injecting two New Zealand rabbits with the purified recombinant XvPer1 protein isolated from polyacrylamide gels and resuspended in phosphate-buffered saline (PBS) pH 7.4. The injection and bleeding of the rabbits were carried out at the UCT Animal Unit. The animals were injected weekly over a 4-week period with a 1 ml antigen emulsion each time (500 µl Freund's incomplete adjuvant + 500 µl antigen resuspension) (Chart and Rowe, 1992). A pre-immune serum sample was taken, as well as weekly serum samples. Successful antibody production was tested by using ELISA (Enzyme-Linked Immunosorbent Assay). The protocol used was adapted from Sambrook et al. (1989). Polysorp ELISA plates (Sigma, UK) were coated with 100 µl of antigen O/N at 4°C. Antigen was diluted 1:2000 – 1:8000. Each dilution was done in triplicate. After O/N incubation the plates were washed 4x with 200 µl of 1xPBS, then blocked for 2 hours with 200 µl of 1% non-fat milk in 1xPBS at RT. The plates were washed again 4x with 200 µl of 1xPBS, which was followed by incubation for 1 hour at RT with 100 µl of primary antibody diluted in 1% non-fat milk in 1xPBS. The plates were washed 4x with 200 µl of 1xPBS, followed by incubation with the secondary antibody [Streptavidin-Alkaline Phosphatase-labelled goat anti-rabbit (Sigma, UK)] at 1:2000 dilution. The plates were incubated at 37°C for 2 hours in a damp environment to prevent evaporation (closed plastic box with damp blotting paper inside). After incubation the plates were washed 4x with 200 µl of 1xPBS and then rinsed 2x with 100 µl of 10% diethanolamine 0.5 mM MgCl₂ (pH 9.5). This was followed by adding 100 µl of pNPP substrate (10 mg *p*-Nitrophenyl phosphate Disodium salt in 10 ml 10% diethanolamine 0.5 mM MgCl₂) to each well and incubating at RT for 30 minutes. After incubation, the reactions were stopped by adding 50 µl of 0.1 M EDTA to each well. The absorbance of each well was read at 405 nm.

After the ELISA was performed, the serum sample demonstrating the highest level on XvPer1-antibody was selected for further experiments. This sample was then purified by isolating the IgG from the serum. This procedure minimises non-specificity during western analysis and other immuno-detection experiments. The method used was adapted from Polson et al. (1964). One volume of serum was mixed with two volumes of borate-buffered saline (2.16 g boric acid, 2.19 g NaCl, 0.7 g NaOH, 620 µl 37%

HCl. Made up to 800 ml with sterile distilled water, pH to 8.6 with NaOH. Adjusted volume with water to 1 L). Crushed PEG 6 KDa was added to the diluted serum to 14% (w/v), gently dissolved by inversion, and the mixture centrifuged at 12000xg for 10 minutes at 4°C. The pellet was redissolved in the original serum volume, using phosphate buffer. PEG was added to 14% (w/v) and dissolved. The solution was centrifuged as before. The pellet containing the IgG was redissolved in half the original serum volume, using phosphate buffer containing 60% (v/v) glycerol, and stored in aliquots at -20°C until use.

3.2.8 Western Blot Analysis

Proteins isolated from *X. viscosa* leaf tissues subjected to dehydration/rehydration stress, heat (42°C), cold (4°C), high light (1500 $\mu\text{molm}^{-2}\text{s}^{-1}$), salinity (100 mM NaCl) and ABA (100 μM) stresses were used for western blot analysis. Quantification of the protein was done using a MICRO-BCA Protein Assay Reagent Kit (Pierce, Rockford, USA). 10 μg of protein from each sampling point was separated by SDS-PAGE. The gels were stained with Coomassie Blue (1 g Coomassie Blue R 250, 45 ml sterile distilled H₂O, 45 ml methanol, 10 ml acetic acid) for viewing but not stained if used for western blotting. For western blotting, after SDS-PAGE, the proteins were transferred electrophoretically onto nitrocellulose membranes (Osmonics, pore size 0.2 μm) using Towbin buffer (25 mM Tris, 192 mM glycine, 20% methanol) with 0.03% SDS added. After transfer, membranes were stained with Ponceau stain [0.1% (w/v) Ponceau S in 5% (v/v) acetic acid] to verify transfer and equal loading of proteins. The membranes were blocked in blotto [5% (w/v) non-fat dried milk in Tris-buffered saline (20 mM Tris pH 7.4, 0.15 mM NaCl)] for 2 hours at room temperature and incubated with 1:2000 dilution of XvPer1 antibodies at 4°C O/N. After 3x10 minutes washes in blotto, the membranes were incubated with 1:5000 dilution of goat anti-rabbit IgG coupled to Horseradish Peroxidase (HRP) (Sigma-Aldrich, UK) for 1 hour. This was followed by 3x10 minutes washes in blotto. The detection was carried out using the ECL detection system (Amersham) following the manufacturer's instructions. The fluorescence was captured on Biomax ML film (Kodak).

3.3 RESULTS

3.3.1 RWC and ψ_w Measurements during Plant Stress treatments

The expression pattern of the *XvPer1*cDNA under various stresses was investigated. *X. viscosa* plants were subjected to dehydration stress, heat (42°C), cold (4°C), high-light (1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$), salinity (100 mM NaCl) and ABA (100 μM) stresses. Sampling of leaf tissue was carried out at specific time points during each stress treatment and RWC and ψ_w were measured. In Figure 3.4(A-F), the RWC and ψ_w of the plant tissues for all treatments, except for the dehydration and heat treatments, remained high. For the heat treatment, the RWC drops to around 80% at 48 hours after the start of the treatment. However, as observed in the dehydration northern blot, *XvPer1* transcript and protein are only induced at around 60% RWC. Therefore, changes in *XvPer1* mRNA transcript and protein levels observed in northern and western analyses were due only to the stress imposed.

3.3.2 Northern Blot Analysis

Northern blot analysis was used to investigate the steady-state mRNA transcript level expression of *XvPer1* in *X. viscosa* leaf tissue subjected to various stress treatments. The *XvPer1* transcript level was higher when the plant was dehydrated from 63% to 4% RWC relative to the fully hydrated state (Fig. 3.5A). *XvPer1* transcripts were present during the early phase of rehydration and disappeared when 92% RWC was reached. The *XvPer1* steady-state mRNA transcript level was also increased by high-temperature stress (Fig. 3.5B). Salinity stress did not appear to affect *XvPer1* abundance, although low levels were observed at days 4-7 (Fig. 3.5C). The *XvPer1* transcript level was increased by cold, with expression starting at day 3 and increasing until day 7 (Fig. 3.5D). A double band was observed in this northern which can be explained either by the relatively poor quality of the extracted RNA or alternatively, by splicing of the transcript. High light stress resulted in an increase in the *XvPer1* steady-state transcript mRNA level (Fig. 3.5E), so did the plant stress hormone ABA (Fig. 3.5F)

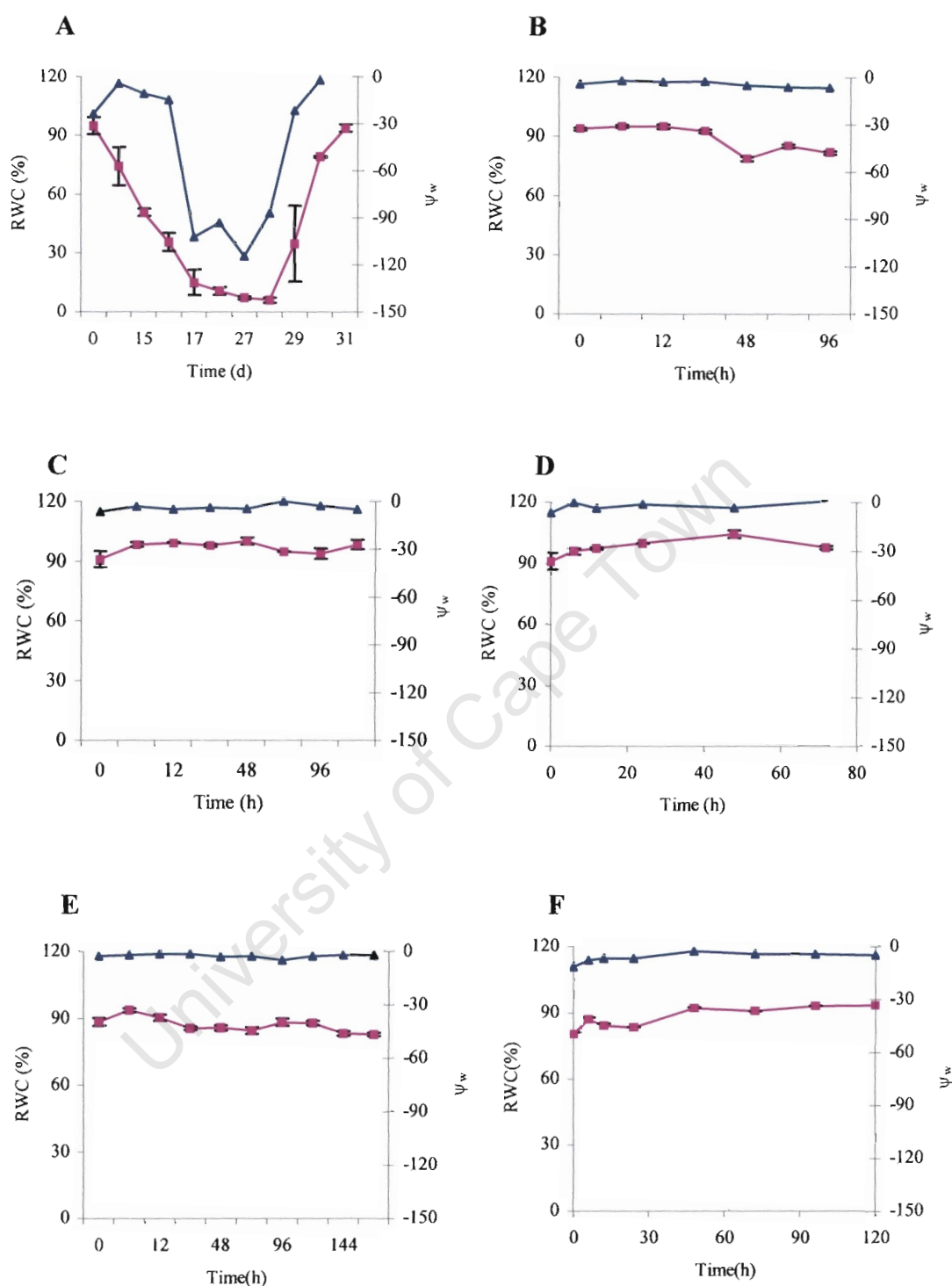


Figure 3.4. A-F Relative water content (■) and water potential (▲) for each stress treatment. A Dehydration/rehydration; B heat (42°C); C NaCl (100 mM); D ABA (100 μ M); E high-light (1500 μ molm⁻²s⁻¹); F cold (4°C). In most cases, the error bars (\pm SE) are wholly contained within the symbol (adapted from Mowla et al., 2002).

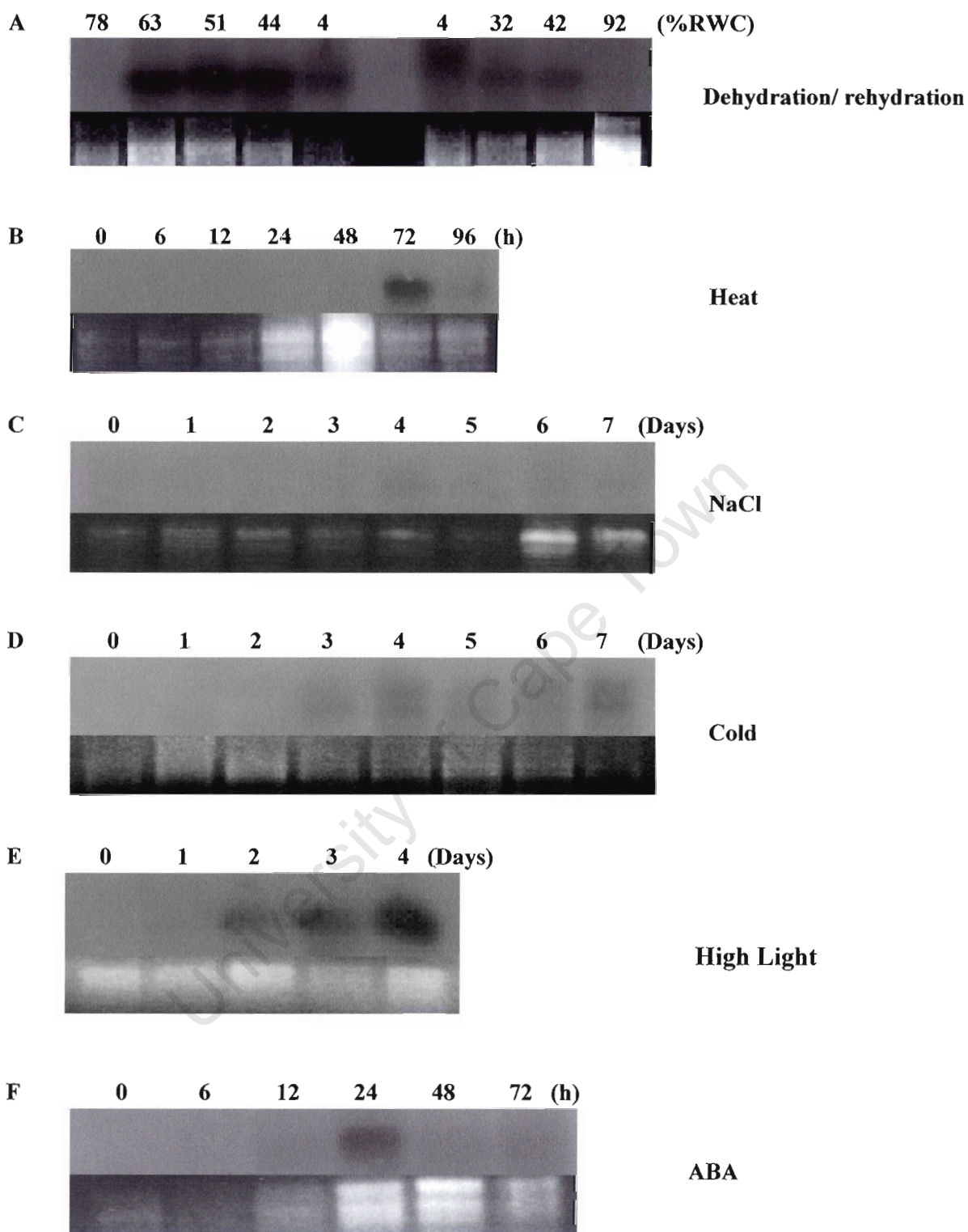


Figure 3.5.A-F Northern blot analysis of *XvPerI* expression under different environmental stress conditions. **A** Dehydration/rehydration; **B** heat (42°C); **C** NaCl (100 mM); **D** cold (4°C); **E** high light (1500 $\mu\text{mol m}^{-2}\text{s}^{-1}$); **F** ABA (100 μM). Lower panels represent ribosomal RNA bands on an EtBr-stained 1.2% agarose gel for comparison of RNA loading in gels (adapted from Mowla et al., 2002).

3.3.3 Expression and Purification of Recombinant XvPer1 Protein

To investigate the expression of the XvPer1 protein under various stresses by western blot analysis, an antibody against XvPer1 had to be generated. The pProEXTM HT prokaryotic expression system (Life technologies, USA) was used to over-express the recombinant XvPer1 protein. The full-length *XvPer1* cDNA was cloned into the pProEXTM HTa plasmid, which was then transformed in *E. coli*. Induction of the recombinant protein expression was achieved by the addition of IPTG to a liquid culture of the transformed *E. coli* growing in the log phase. Figure 3.6 illustrates an SDS-PAGE of total proteins isolated from 1 ml of bacterial culture harvested hourly after induction, as well as O/N.

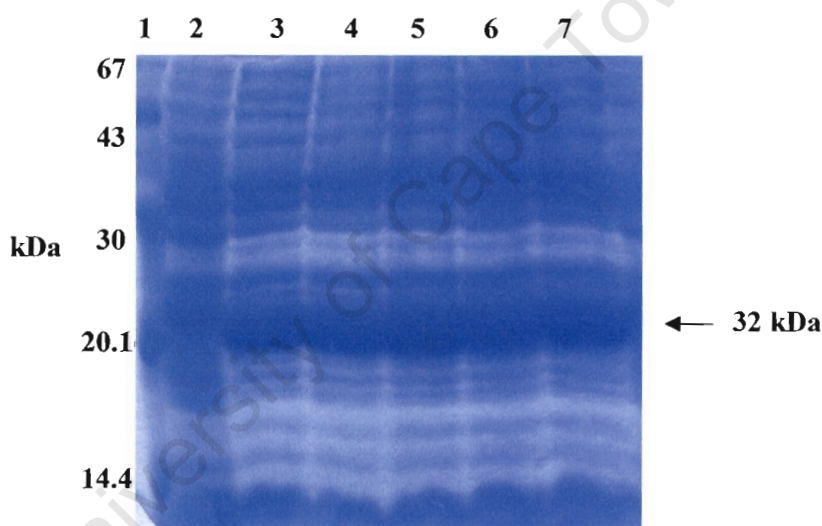


Figure 3.6. Analysis of XvPer1 recombinant protein induction with IPTG over time. Proteins from DH5 α *E. coli* cells transformed with pProEX-XvPer1 were electrophoresed on a 12.5% SDS-PAGE. Lane 1: Low Molecular Weight marker; lane 2: uninduced cells; lanes 3-7: induction after 1, 2, 3, 4 hours and overnight respectively. A protein of approximately 32 kDa was being induced (24 kDa + His tag + ~ 30 amino acids upstream of cloning site).

A very clear over-expressed protein band is produced soon after induction (1 hour), positioned at 32 kDa, which is the approximate expected size of the recombinant protein. Some expression is also evident even before addition of IPTG. This is to be expected as the *trc* promoter is known to be 'leaky'. However, high induction of the promoter after addition of IPTG is clear.

The expressed XvPer1 protein was fused to a 6 histidine sequence (His)₆ for affinity purification. To purify the recombinant protein, the QIAexpressTM expression system (Qiagen, Germany) was used. Soluble protein lysates were loaded onto a nickel-nitriloacetic acid (Ni-NTA) affinity column which selectively binds the 6xHis-tagged protein. After several washing steps, the purified recombinant protein was eluted. Figure 3.7 illustrates an SDS-PAGE of the progressive steps involved in the purification of recombinant XvPer1 by use of the Ni-NTA column. A very prominent single band can be observed in the elution steps, which is of the expected size of 32 kDa. A comparatively small amount of protein was eluted in the washing steps as well. This was probably due to a very high level of His-tagged protein bound to the matrix.

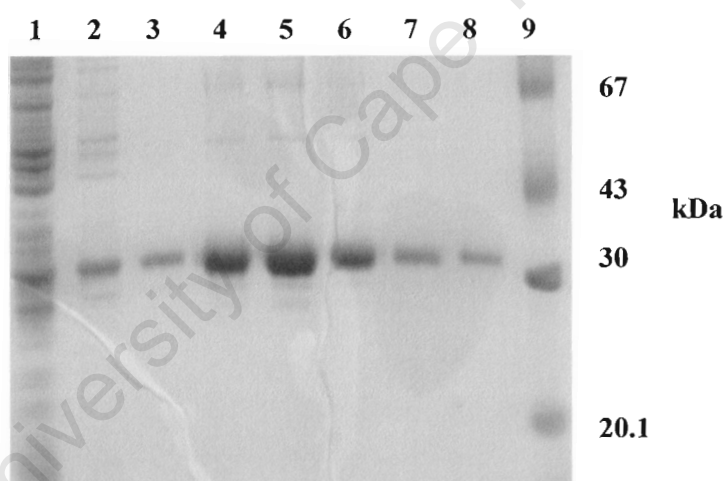


Figure 3.7. SDS-PAGE representing the batch purification of His-XvPer1 using the Ni-NTA affinity column. Aliquots from the purification of His-XvPer1 were analysed on a 12.5% SDS-PAGE. Lane 1: Ni-NTA column flow-through; lane 2: buffer A wash; lane 3: buffer B wash; lanes 4-8: elution fractions; lane 9: Low Molecular Weight marker with sizes indicated in the right margin.

3.3.4 The Production of XvPer1 Antibodies and IgG Purification

The production of polyclonal XvPer1 antibodies was achieved by injecting purified recombinant His-tagged XvPer1 proteins into two rabbits. Weekly injections were performed on the animals over a period of 4 weeks and serum samples were collected each week. To assess the animals' immune responses to the antigen, ELISA was

carried out on each serum sample from each animal. Figure 3.8 illustrates a plot of OD₄₀₅ v/s dilutions times for each serum sample. Since ELISA on each dilution was carried out in triplicate, an average of the OD₄₀₅ was taken. Rabbit B had a higher immune reactivity to XvPer1, especially at week1 and week 4. For further immuno-detection experiments, the Bleed 4 serum from rabbit B was selected and the IgG was purified.

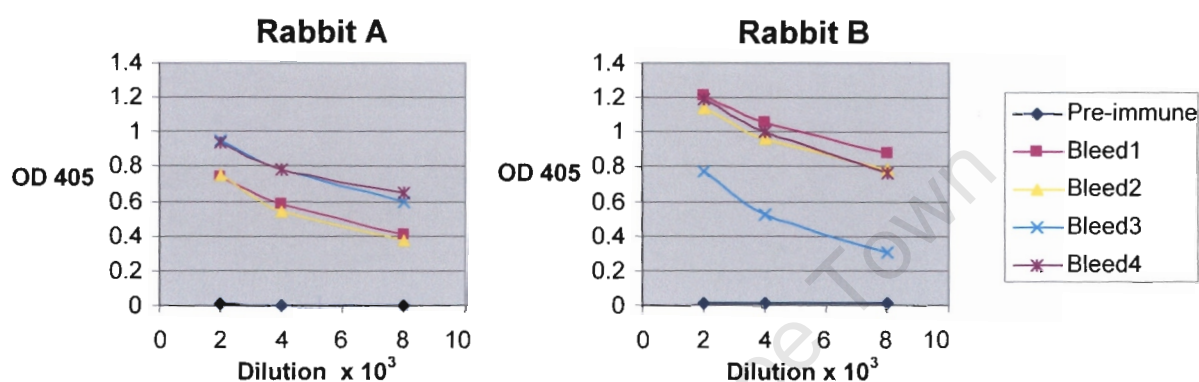


Figure 3.8. ELISA of serum samples obtained from rabbits A and B inoculated with His-XvPer1. Bleed 1 represents serum obtained one week after inoculation. Bleed 2 represents serum obtained two weeks after inoculation, and so on. The dilution time for each sample was done in triplicate and the average OD taken.

3.3.5 Western Blot Analysis

Western blot analysis was used to investigate the accumulation of the XvPer1 protein in *X. viscosa* in response to dehydration/rehydration, high temperature (42°C), low temperature (4°C), high light (1500 $\mu\text{molm}^{-2}\text{s}^{-1}$), salinity (100 mM NaCl) and in response to external application of the stress hormone ABA (100 μM). Protein was isolated from *X. viscosa* leaf tissue collected at specific time points for each stress treatment and 10 μg of each sample was separated by SDS-PAGE. The proteins were then transferred onto nitrocellulose membranes which were probed with XvPer1 antibody using an ECL detection system.

Under dehydration stress, XvPer1 protein is absent at 74% RWC but present at 51% RWC (Fig 3.9A). The protein level then stays relatively constant until the end of dehydration at 6% RWC. Upon rehydration, XvPer1 stays in relatively high amounts

up to 80% RWC and then disappears at 95% RWC when the plant is almost fully hydrated. XvPer1 is not induced very early on under high temperature stress (Fig 3.9B). There is no evidence of protein expression right up to 48 hours after start of the treatment. At 72 hours XvPer1 is present, and its level has increased at 96 hours. Under salinity stress, XvPer1 expression was detected at 48 hours and 72 hours but not at 96 hours (Fig 3.9C). ABA substantially induced XvPer1 expression with high levels accumulating at 48 hours and 72 hours (Fig 3.9D). No expression was detected under cold and high-light stresses (Data not shown).

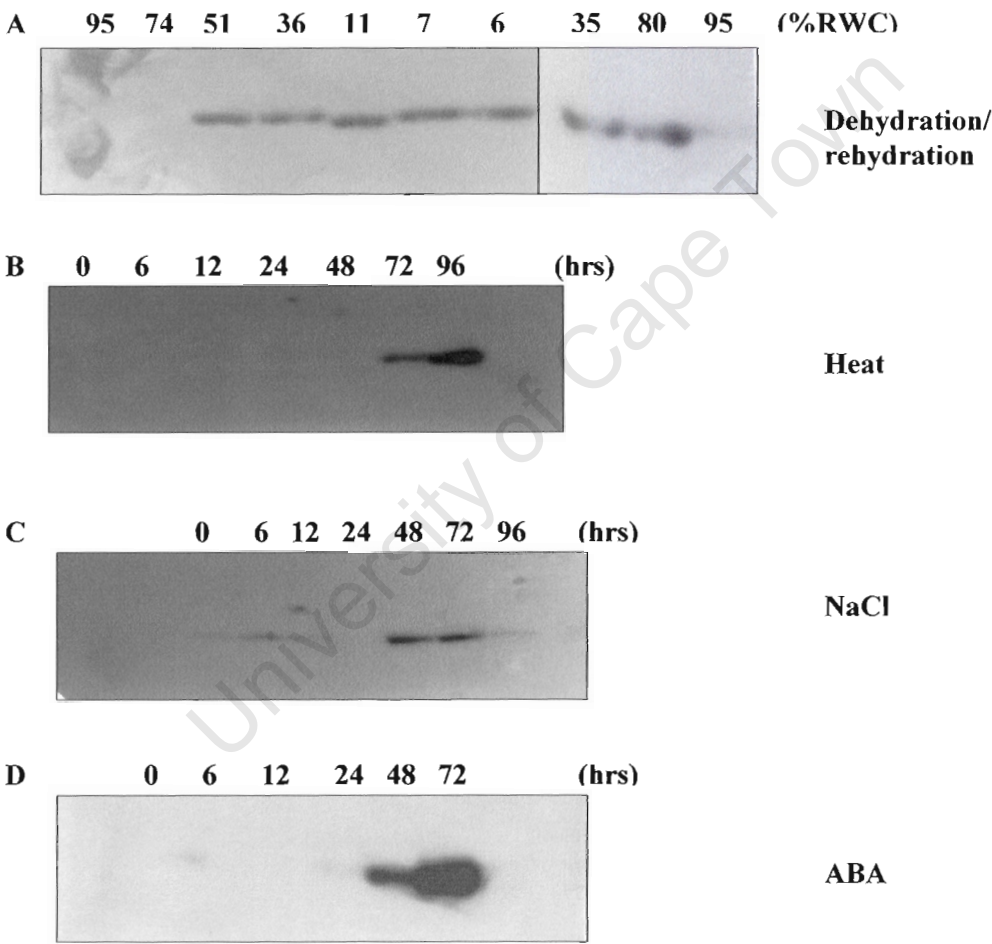


Figure 3.9.A-D Western blot analysis of *X. viscosa* exposed to different environmental stress treatments. **A** Dehydration/rehydration; **B** heat (42°C); **C** NaCl (100 mM); **D** ABA (100 µM) (adapted from Mowla et al., 2002).

3.4 DISCUSSION

Northern blot analysis was used to investigate the expression of the steady-state mRNA transcript of *XvPer1* in *X. viscosa* leaf tissues subjected to various artificially induced environmental stresses. These were dehydration and rehydration, heat (42°C), cold (4°C), salinity (100 mM NaCl), high light intensity (1500 $\mu\text{molm}^{-2}\text{s}^{-1}$), as well as the stress-hormone ABA (Mowla et al., 2002). *XvPer1* was initially isolated by differential screening of a cDNA library constructed from RNA isolated from dehydrating *X. viscosa* leaves. It has been shown that many genes induced by drought are also induced by other stresses such as salinity and cold (Zhu et al., 1997). To be sure that the change in the level of the *XvPer1* transcript observed was indeed as a result of the stress imposed, the RWC and ψ_w of the leaf tissues subjected to the stresses were monitored at each sampling point. Apart from the dehydration/rehydration treatment, the RWC and ψ_w of the plants subjected to all the treatments remained high enough not to impose additional stress besides the intended one (Fig. 3.4A-F).

The *XvPer1* steady-state mRNA transcript and protein abundance when the plant was dehydrated increased from 63% to 4% RWC relative to the fully hydrated state (Fig. 3.5A & 3.9A) (Mowla et al., 2002). During the early phase of rehydration both the transcripts and proteins were still present, but disappeared when 92% RWC was reached. *X. viscosa* is a poikilochlorophyllous plant and loses its chlorophyll and dismantles its photosynthetic system while in a dehydrated state (Sherwin and Farrant, 1997). In such a state, protection of the plant cell membrane is crucial for survival. *XvPer1* might play a role in protecting the membranes against lipid peroxidation, caused by ROS, by the removing these highly oxidative and unstable molecules. When *X. viscosa* was exposed to high temperature stress the *XvPer1* steady-state mRNA transcript and proteins levels were also increased (Fig. 3.5B & 3.9B). However, the transcript and proteins started to accumulate only 48 hours after the stress had started. Transcript accumulation was observed at 72 hours after start of treatment, but significantly lowered at 96 hours. The protein levels kept on increasing at 96 hours. This result suggests that *XvPer1* is not involved in the initial stages of the plant's response to heat stress. Heat stress affects most cellular processes as it leads

to the denaturation of proteins, the inactivation of enzymes and damage to lipid membranes brought on by the production of ROS (Munro and Pelham, 1985). To tolerate and survive heat stress, the plant requires the ability to repair the damages incurred by heat. It is expected therefore that *XvPer1* would have a role to play in either the detoxification of ROS, or the protection of the membrane lipids.

Salinity stress did not appear to affect *XvPer1* abundance, although low levels were observed at days 4-7 (Fig. 3.5C). However, the XvPer1 protein level was increased 48 hours after the commencement of the stress and the level stayed relatively constant at 72 hours, before decreasing at 96 hours after start of the treatment (Fig. 3.9C). Applications of high exogenous salt concentrations cause an imbalance in the ions in the plant cells leading to ion toxicity, osmotic stress and production of ROS (Hasegawa et al., 2000). Like for heat stress, during salinity stress XvPer1 might be involved in the scavenging of ROS and hence maintaining membrane integrity by controlling lipid peroxidation caused by ROS.

The *XvPer1* transcript was increased by cold, with expression starting at day 3 and increasing until day 7 (Fig. 3.5D). However, the XvPer1 protein was not detected over such period of time (data not shown). In its natural environment, which is often at very high altitudes, the *X. viscosa* plant is often exposed to very low temperatures, even below freezing. Therefore it might be possible that an imposed low temperature stress of 4°C is not stressful enough for the conditions necessary for the translation of the XvPer1 protein to be induced in the plant cell. Cellular triggers, such as transcription factors, responsible for the induction of the mRNA transcript might be induced under those conditions, but not those responsible for protein induction.

High light intensity strongly induced the *XvPer1* steady-state mRNA transcript level (Fig. 3.5E). The transcript started accumulating 48 hours after start of the stress and kept on increasing until the end of the stress at 96 hours. However, as for cold stress, protein induction was not observed over the period of time of the stress (data not shown). Again, this could be due to the fact that *X. viscosa* is an extremophile and is regularly exposed to high light intensities in its natural habitat. The trigger for the *XvPer1* transcript accumulation might be present, but not the trigger for protein translation. High light intensity can result in the formation of ROS. If those free

radicals are not quenched, DNA damage and lipid peroxidation occurs. At earlier stages of high light stress, *X. viscosa* probably has other mechanisms in place for protection against cellular damage such as the accessory pigments like the xanthophyll cycle pigments and β -carotene, as well as via the glutathione-ascorbate pathway (Foyer et al., 2005). The 1-Cys peroxiredoxin enzyme probably comes into play later during the stress.

The plant stress hormone ABA also induces *XvPer1* expression (Fig. 3.5F). The steady-state mRNA transcript starts accumulating at 12 hours after start of the treatment and increases at 24 hours. However, at 48 and 72 hours, when the treatment was ended, transcripts had decreased again to almost non-detectable levels. In contrast to this, the *XvPer1* protein was not detected up to 24 hours after start of the treatment (Fig. 3.9D). However, the protein accumulated at 48 hours and increased at 72 hours. This is a clear indication that for the 1-Cys peroxiredoxin enzyme in *X. viscosa*, the transcription of the mRNA transcripts and the translation into protein do not take place simultaneously at all times. The presence of the mRNA transcript is not an indication of the presence of the protein as well. And similarly, that absence of the mRNA transcript does not necessarily indicate the absence of an active protein. However, this observation might also be explained simply by the difference in the level of sensitivity of the two methods.

ABA is involved in the generation of many stress-inducible genes and is required for changes in gene expression in response to water deficit stress (Bray, 1997). The analysis of promoters and mutants having altered responses to ABA provided evidence of multiple signalling pathways and changes in gene expression that are ABA-dependent and/or -independent (Bray, 2002). Analysis of the promoter of *AtPer1*, the Arabidopsis orthologue, identified an ABRE (ABA response element), and an ARE (antioxidant response element) (Haslekas et al., 1998). It is therefore very likely that *XvPer1* expression is ABA-dependent. Ingle et al. (unpublished) used splinkerette PCR (Devon et al., 1995) to isolate ~2 kb of upstream sequence from the *XvPer1* gene in the *X. viscosa* genome. Bioinformatics analysis revealed differences between the promoter sequence from *X. viscosa* and the promoter structure of *AtPer1*. Although both promoters had similar elements, their arrangement and numbers

differed. Both upstream sequences contained ABRE, ARE, ACGT, DFBF (Bzip site), DRE (C-element), MYB, MYC, RY repeat (endosperm specific) and salicylic acid/jasmonate responsive cis-elements.

From the data accumulated in the northern and western analysis studies, it is clear that the *X. viscosa* 1-Cys peroxiredoxin is a stress-inducible enzyme. The transcripts of all previously isolated plant 1-Cys Prx were always specific to the aleurone layer and the embryo of seeds (Aalen et al., 1994; Goldmark et al., 1992; Haslekas et al., 2003a). This led the authors to postulate that the protein performs a protective function in the only two tissues that survive desiccation.

XvPer1 is absent from healthy unstressed plants, but is transcribed as soon as the plant is exposed to abiotic stresses. *X. viscosa*, a resurrection plant, can behave like a seed, i.e., it can stay in a dry but viable state for long periods of time when unfavourable conditions prevail without sustaining major tissue damage. It therefore has certain seed-specific properties it is not surprising that it should express certain 'traditionally' seed-specific genes in times of stress.

CHAPTER FOUR

IMMUNOLocalIZATION OF XvPER1

SUMMARY

Hydrated and dehydrated *X. viscosa* leaf tissues were embedded in paraffin. Sections were cut and incubated with 1:200 dilutions of anti-XvPer1 serum and pre-immune serum. The immunoreactivity was visualised using fluorochrome tagged secondary goat anti-rabbit IgG antibody at a dilution of 1:1000. The fluorescence was viewed under oil emersion with an inverted fluorescent microscope. XvPer1 was localized in the nucleus of dehydrated but not hydrated *X. viscosa* leaf cells. For further confirmation of the result, an alternative method of tissue preparation and immunolocalization was used. Water-treated (control) and ABA-treated leaf tissue from *X. viscosa* tissue culture plantlets were fixed and embedded in LR-white. Sections were incubated with 1:100 dilution of anti-XvPer1 serum followed by incubation with 1:50 dilution of alkaline phosphatase-labelled goat anti-rabbit. The immunoreactivity was detected using the SIGMA *FAST* Red TR/Naphthol AS-MX Phosphate tablets which detect alkaline phosphatase activity to produce an intense red stain. This was visualised using the light microscope. XvPer1 was found to be localized mainly in the nucleus of ABA treated and not water treated (control) *X. viscosa* tissue. XvPer1 was also weakly detected in the cytoplasm. A histone specific antibody (H3) and an ascorbate peroxidase (APX) antibody were used as controls. In both types of immunolocalization studies, western blot analyses were performed on the treated plant tissues to confirm the expression of the XvPer1 protein prior to immunolocalization.

4.1 INTRODUCTION

The plant 1-Cys Prx genes *pBSI28* from brome grass (Goldmark et al., 1992), *Per1* from barley (also know as B15C; Aalen et al., 1994), *AtPer1* from Arabidopsis (Haslekas et al., 1998), and *FePer1* from buckwheat (*F. esculentum*; Lewis et al., 2000) are all expressed in developing seeds and are maintained at high levels in mature dry seeds. During imbibition, the transcripts disappear in germinating seeds, but in dormant non-germinating seeds, the transcript levels are maintained or even transiently up-regulated. This expression pattern led Stacy et al. (1996) to suggest that

1-Cys Prx is involved in the maintenance of seed dormancy. However, further experiments questioned this. High expression levels of *AtPer1* in the non-dormant *aba1* mutant seeds indicated that maintenance of the protein alone is not sufficient to induce dormancy in Arabidopsis (Haslekas et al., 1998). Furthermore, tobacco seeds (*Nicotiana tabacum*) expressing the rice 1-Cys Prx (*RIC-Prx*) germinated as efficiently as wild-type seeds (Lee et al., 2000).

A second putative function, which is more plausible, has also been suggested, which is in the facilitation of desiccation tolerance during the late stages of seed development (Stacey et al., 1996). ROS accumulates in high levels during desiccation and is highly damaging (LePrince et al., 1994). ROS have deleterious effects on membranes, proteins and DNA. The Barley PER1 has been localized to the nucleus of cells of the embryo and aleurone layer, although a weak signal was also detected in the cytoplasm (Stacy et al., 1999). Therefore a role in protection of nucleic acid against ROS has been suggested. In this chapter, the subcellular localization of XvPer1 in *X. viscosa* will be described.

4.2 MATERIALS AND METHODS

4.2.1 Plant Material and Growth Conditions

For immunofluorescence studies, *X. viscosa* plants were collected and cultivated as described in section 2.2.1.

For chromogenic immunodetection, *X. viscosa* plantlets were grown from seed (Silverhill Seeds, South Africa) under tissue-culture conditions. Seeds were sterilised by washing in 70% ethanol for 5 minutes, followed by washing in 10% sodium hypochlorite solution with 0.02% Triton X-100 for 10 minutes, followed by 5 washes with sterile distilled water. The seeds were resuspended in 0.1% agar, then transferred to a larger sterile tube, making up the volume of 0.1% agar to 2.5 ml. When ready to plate, 2.5 ml of cooled (50°C) 0.75% agar was added to the seed-agar mix, agitated, and poured onto a plate containing plant nutrient agar medium (PNS) supplemented with sucrose (5 mM KNO₃, 2 mM MgSO₄·7H₂O, 2 mM Ca(NO₃)₂·4H₂O, 20 mM Fe EDTA, 1x micronutrients, 0.5% sucrose, 0.75% agar). Micronutrient stock (1000x) was composed of 70 mM H₃BO₄, 14 mM MnSO₄·H₂O, 0.5 mM CuSO₄·5H₂O, 1 mM

ZnSO₄·6H₂O, 0.2 mM Na₂MoO₄, 10 mM NaCl, 0.01 mM CoCl₂·6H₂O. The plates were incubated in the dark at 4°C for 4 days before being transferred to a growth chamber with 16/8 hours day/night cycle, at 25°C, with a light intensity of 200 $\mu\text{mol m}^{-2}\text{s}^{-1}$. Germinated seeds were transferred onto fresh media into bigger tissue culture bottles. *X. viscosa* plantlets which were 7-10 cm in height and had developed roots were selected for stress treatments.

4.2.2 Plant Stress Treatment

For immunofluorescence, hydrated leaf tissue was excised from the fully hydrated plant. For the dehydration treatment, a *X. viscosa* plant was left unwatered for 2 weeks until it had reached an air-dried state. Leaves were excised and the RWC calculated as described in section 3.2.2.

For chromogenic immunodetection, the aerial parts of two *X. viscosa* plantlets were sprayed with 100 μM ABA and the pots were covered with aluminium foil to keep away light as ABA is light sensitive. After 72 hours, the leaves were harvested for protein isolation and for fixation. Two control plants were treated in exactly the same way but sprayed with water instead of ABA.

4.2.3 Verification of XvPer1 Expression by Western Blot Analysis

Soluble proteins were extracted from the dehydrated tissue and ABA treated tissues as described in section 3.2.3. Western blot analysis was performed as described in 3.2.8.

4.2.4 Tissue Fixation and Embedding

For immunofluorescence detection, hydrated and dehydrated *X. viscosa* leaf tissues were cut into 5mm² pieces and fixed overnight at 4°C in 4% (w/v) paraformaldehyde in 0.1 M Sorenson's phosphate buffer (pH 7.2). Specimens were then dehydrated through a graded alcohol series (50%, 70%, 95% and 100% ethanol, 100% isopropanol, 100% butanol) and embedded in paraffin wax at 60°C overnight. The protocol was adapted from Van der Willigen (2001).

For chromogenic immunodetection, leaf tissues from *X. viscosa* plantlets were cut in pieces (~2 mm²) and fixed in 4% paraformaldehyde and 0.1-0.5% glutaraldehyde in

0.05 M PB buffer pH 7.2 by vacuum infiltration at 28 mbar (Edwards E2M5 High vacuum pump SPI, USA). Fresh fixative was replaced after vacuum infiltration and the samples incubated for 2 hours at RT, followed by O/N incubation at 4°C. Thereafter the samples were washed in 0.05 M PB buffer for 2x10 minutes with rotation at RT. This was followed by dehydration through an alcohol series (10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100% ethanol). The samples were infiltrated with 2:1 ethanol:LR white for 2 hours at RT, followed by 1:1 ethanol:LR white for 2 hours, followed by 1:2 ethanol:LR white for 2 hours and finally with 100% LR white for 2 hours. Thereafter the resin was replaced twice a day for 2 days (O/N at 4°C). Polymerisation was carried out in a pre-warmed oven at 50°C for 24 hours without oxygen (nitrogen flux).

4.2.5 Immuno-Detection

Immunofluorescence detection

Transverse sections (10 µm) were cut using a Leitz (Vienna, Austria) rotary microtome and fixed onto APTES- (3-aminopropyltriethoxysilane) coated glass microscope slides by heating to 60°C for 1 hour. After dewaxing with xylol and rehydrating through an ethanol gradient, the sections were washed with PBS (pH 7.4) and blocked with 1% BSA (w/v) in PBS for 1 hour in a moist chamber (in a closed plastic box with moist paper towels) at RT. Sections were incubated with either primary antibody (anti-XvPer1, 1:200 dilution) or preimmune-serum (1:200 dilution) overnight at 4°C in a moist chamber. Thereafter, the sections were jet washed (using Pasteur pipette) with 0.1% Triton X-100 in PBS and then rinsed for a further 15 minutes in PBS buffer before incubating in the fluorochrome tagged secondary goat anti-rabbit IgG antibody (Alexa fluor 568, Molecular Probes, Oregon USA) at 1:1000 dilution for 2 hours in a moist chamber at RT in the dark. The slides were then thoroughly washed with PBS containing 0.1% Triton X-100. Sections were mounted with ProLong Antifade (Molecular Probes, Oregon, USA) and viewed with an inverted fluorescent microscope (Nikon, Tokyo, Japan). The approximate absorption and fluorescence emission maxima for Alexa fluor 568 are 578 nm and 603 nm respectively.

Chromogenic immunodetection

1 μ m thick sections were cut using an ultramicrotome (Reichert-Jung, USA) and were fixed on acid-washed and poly-lysine coated multi-well glass microscope slides (Merck, USA) at 42°C O/N. The sections were blocked with PBSTA (0.01 M PBS, 1% BSA, 0.01% Tween-20, 20 mM Na_3Na) with 10% goat serum (GS) for 30 minutes at RT. Thereafter the sections were incubated with primary antibody diluted in PBSTA with 1% GS and kept O/N at 4°C in a humidity chamber. Three different primary antibodies were used: anti-XvPer1, anti-H3, and anti-APX, each at three different dilutions: 1:500, 1:100 and 1:20. The H3 antibody was derived from histone calf thymus type III (Sigma, UK) raised in rabbit (Freeman and Ougham, 1994). The APX antibody used was raised in the following manner: protein sequences of cytosolic, peroxisomal and plastidic APX were aligned using Genetics Computer Group (GCG) program PILEUP, allowing identification of sequences conserved in cytosolic but absent in plastidic APX isoforms (Mittova et al., 2004). A peptide corresponding to the N-terminus of maize cAPX (Q41772) (6-PTVNEDYLKAVDKAKRKLRGLIAEKNCA-33) was synthesized and coupled to keyhole limpet haemocyanin by Sigma Genosys (Pampisford, Cambs, UK). This antibody is specific to the cytosol, and absent in the chloroplast. After incubation, the sections were rinsed with 1 jet of PBST (0.01 M PBS with 0.01% Tween-20) followed by immersion in Couplin jars with PBST for 2x10 minutes each. The sections were then blocked in PBSTA with GS (10%) for 30 minutes at RT before incubating with goat anti-rabbit alkaline phosphatase secondary antibody diluted 1:50 in PBSTA with GS (1%) for 2 hours at RT in a humidity chamber. After incubation the sections were rinsed with 1 jet of PBST followed by immersion in Couplin jars with PBS for 2x10 minutes. For detection, the substrate (SIGMAFAST™ Fast Red TR/Naphthol AS-MX tablets; Sigma, UK) was added to the sections and the colour development was monitored under the light microscope. The reactions were stopped by submerging the slides in distilled water.

4.3 RESULTS

4.3.1 Western Blot Analysis of Dehydrated and ABA-treated *X. viscosa* Leaf Tissue

A mature *X. viscosa* plant was allowed to dehydrate by withholding water from the soil. The RWC of the air-dried plant was 11%. Before attempting immunolocalization on the dehydrated *X. viscosa* leaf tissue, it was important to verify that the XvPer1 protein was expressed in the tissue. Total soluble proteins were isolated from a section of the same tissue used for fixation and embedding in paraffin. After separation by SDS-PAGE, the proteins were transferred onto nitrocellulose membrane and then probed with the anti-XvPer1 antibody using an ECL detection system. The same procedure was carried out with leaf tissue from ABA treated *X. viscosa* plantlets. Figure 4.1A shows the western blot analysis on dehydrated tissue where XvPer1 expression is clear in the dehydrated leaf tissue. As a positive control, protein from *X. viscosa* treated with heat stress (42°C) for 96 hours was used (protein sample used in experiment illustrated in section 3.3.5). Figure 4.1B shows the western blot analysis on ABA and treated *X. viscosa* tissue. No protein was detected in the water treated sample, but a very clear expression of XvPer1 can be observed in the ABA treated sample.

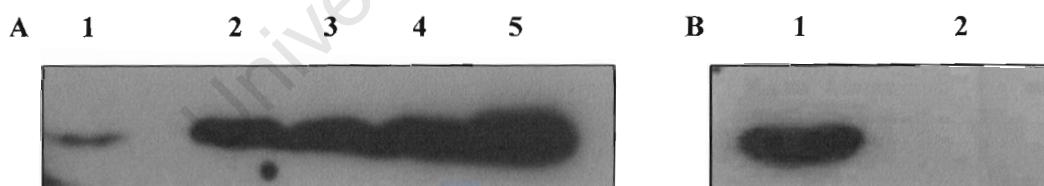


Figure 4.1. Western blot analysis of dehydrated and ABA treated *X. viscosa* leaf tissue probed with anti-XvPer1 using the ECL detection system. **A**-Lane 1: 10 μ l of protein from heat treated leaf tissue (42°C) for 96 hours (used as a positive control); lanes 2 -5: 5 μ l, 10 μ l, 15 μ l & 20 μ l of protein isolated from dehydrated leaf tissue. **B**-Lane 1: 10 μ g of protein from leaf tissue sprayed with 100 μ M ABA and harvested after 72 hours; lane 2: 10 μ g of protein from leaf tissue sprayed with sterile water (control experiment) and harvested after 72 hours.

4.3.2 Immunolocalization of XvPer1

Immunofluorescence detection in dehydrated leaf tissue

A putative NLS was identified in the XvPer1 amino acid sequence (see section 2.3.1). Sections were probed with 1:200 dilutions of either preimmune-serum or anti-XvPer1. Figure 4.2 illustrates the fluorescence observed from hydrated and dehydrated leaf tissues when probed with preimmune-serum as a control. Compared to the anti-XvPer1 probed sections (Fig. 4.3), very low or almost no fluorescence was observed. Figure 4.3 shows sections from hydrated and dehydrated leaves of *X. viscosa* and clearly shows that XvPer1 is localised to the nucleus of dehydrated *X. viscosa* leaf tissue. Thymol blue staining in the hydrated section was used to locate nucleus-containing sections. No fluorescence was detected in hydrated tissue, which confirms XvPer1 as being a stress-inducible protein. Although a hint of red fluorescence can be observed in Fig 4.3 (d), XvPer1-probed hydrated sections without thymol blue staining showed only relatively faint and non-specific fluorescence (data not shown).

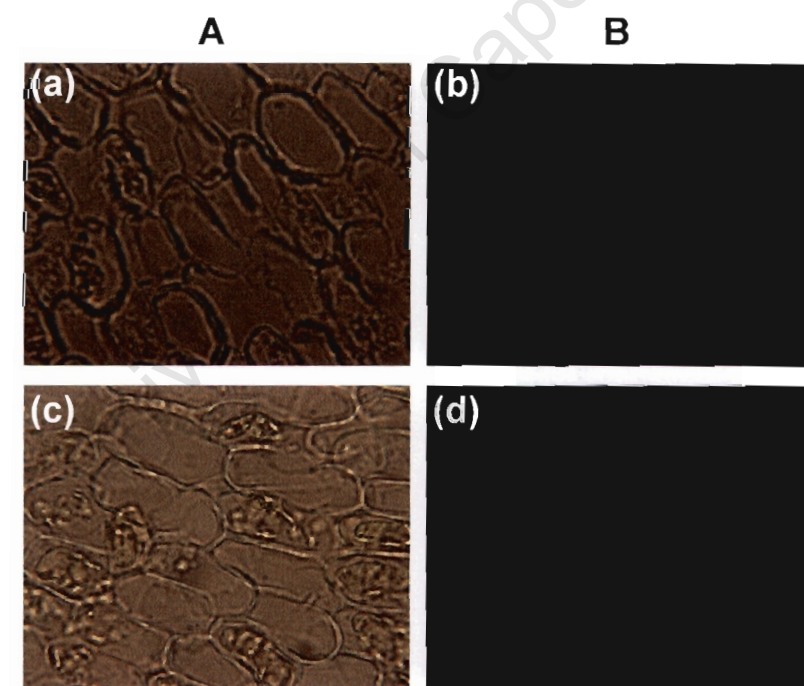


Figure 4.2. A and B Control experiment for subcellular immunofluorescence localization of XvPer1 in hydrated and dehydrated *X. viscosa* leaf cells. Paraffin-embedded sections were incubated in pre-serum and goat anti-rabbit IgG secondary antibody conjugated to Alexa fluor 568 for detection by inverted microscopy. Red fluorescence indicates an XvPer1-specific reaction. **A:** Transverse sections as seen under the inverted light microscope; **B:** the same sections under fluorescent light. *a* and *b*: dehydrated *X. viscosa* leaf cells incubated with pre-serum. *c* and *d*: hydrated *X. viscosa* leaf cells incubated with pre-serum. 5,000x

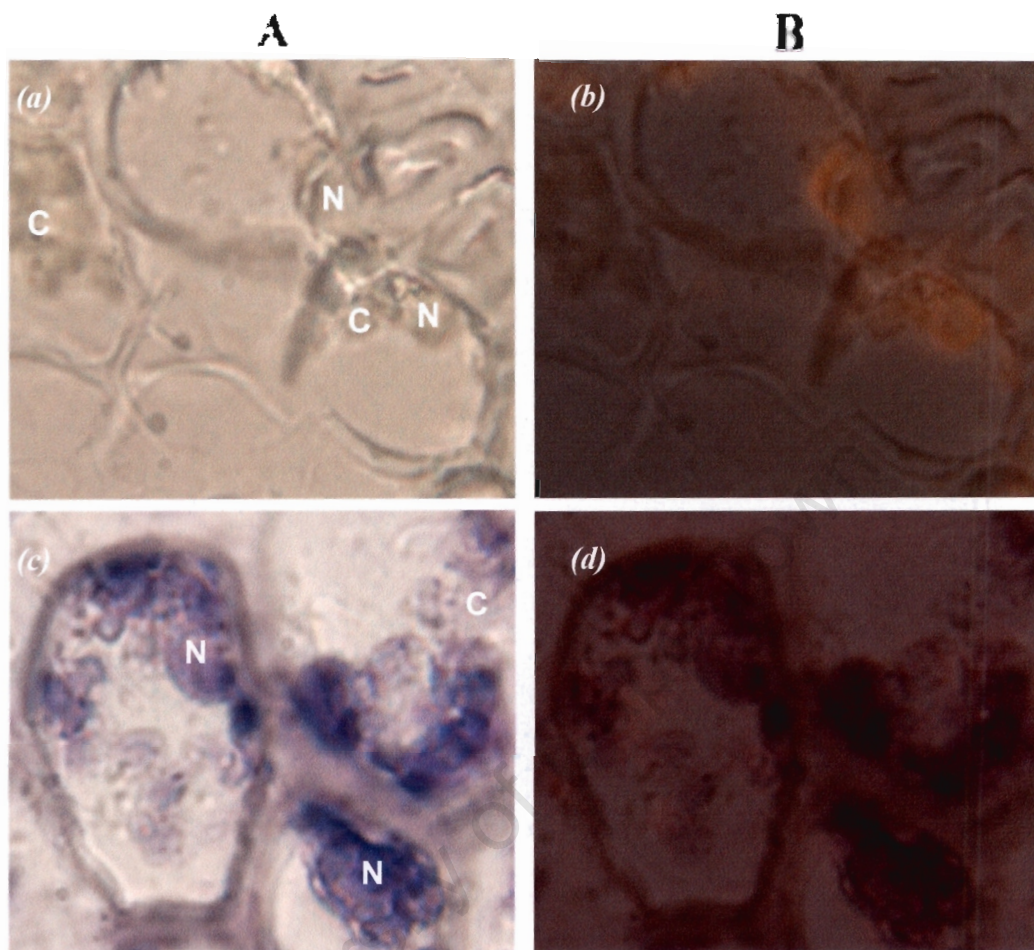


Figure 4.3. A and B. Subcellular immunofluorescence localization of XvPer1 in hydrated and dehydrated *X. viscosa* leaf cells. Paraffin-embedded sections were incubated in XvPer1 antiserum and goat anti-rabbit IgG secondary antibody conjugated to Alexa fluor 568 for detection by inverted microscopy. Red fluorescence indicates an XvPer1-specific reaction. **A:** Transverse sections as seen under the inverted light microscope; **B:** the same sections under fluorescent light. (a) and (b): dehydrated *X. viscosa* leaf cells incubated with anti-XvPer1; (c) and (d): hydrated *X. viscosa* leaf cells, stained with thymol blue, incubated with anti-XvPer1. N: Nucleus, C: chloroplast. 10,000x (adapted from Mowla et al., 2002).

Chromogenic immunodetection in ABA-treated leaf tissue

Because XvPer1 has a putative nuclear location, the anti-H3 was used to verify that a nuclear-localized protein could indeed be detected using this method. Figure 4.4 shows that immunocytochemistry of a nuclear-localized protein is possible using this technique and this type of tissue.

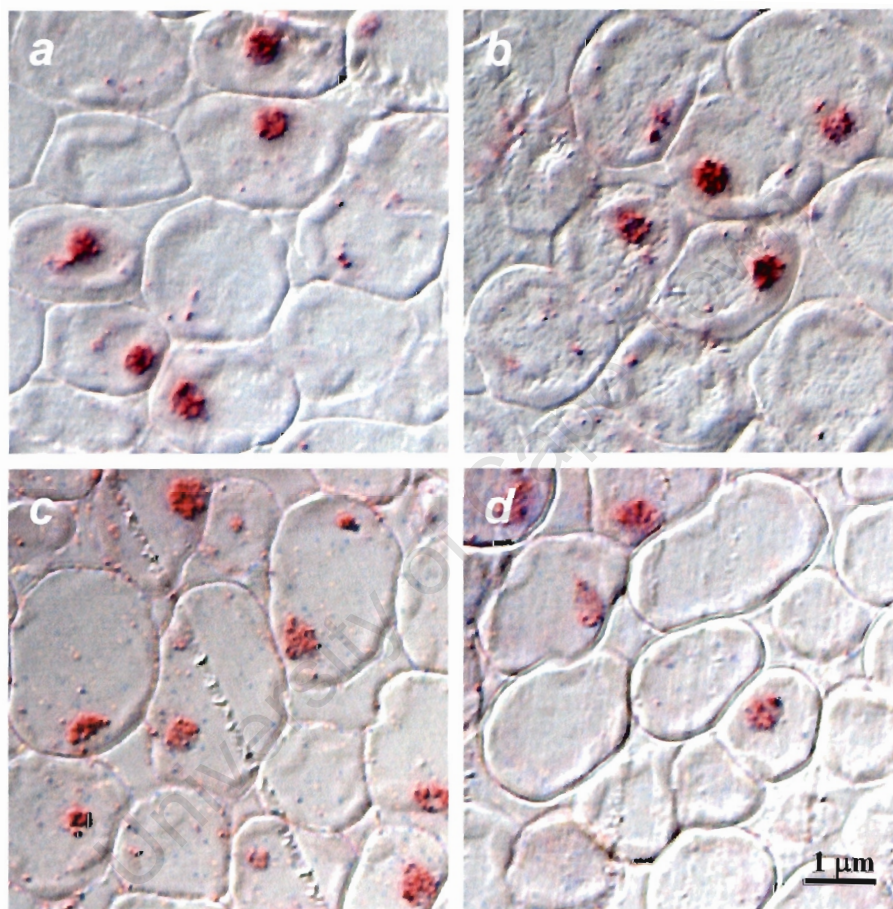


Figure 4.4. Immunolocalization of the nuclear localized H3 protein in water-treated (*a* and *b*) and 100 μ M ABA-treated (*c* and *d*) *X. viscosa* leaf tissue using the Fast-Red precipitation technique (Sigma, UK).

The APX antibody was used as a second control antibody. Since this enzyme is known to be cytosolic, this control was used to verify that cytoplasmic content had been adequately fixed into place and no contamination with the vacuole or the nucleus had occurred. This would ensure that the result obtained when probing the same tissue with the XvPer1 antibody is genuine. Figure 4.5 shows the result obtained when

probing with a 1:100 dilution of the APX antibody. Red staining is clearly visible only in the periphery of the cells, close to the cell membrane. No specific precipitation of the substrate was observed in the vacuole in both the water treated and the ABA treated tissue samples. No red staining of apparently nuclear material similar to the ones observed in the anti-H3 probed samples were observed here. This suggests that it is highly unlikely that either the vacuole or the inside of the nucleus got contaminated with cytoplasmic contents during the fixation and embedding procedures.

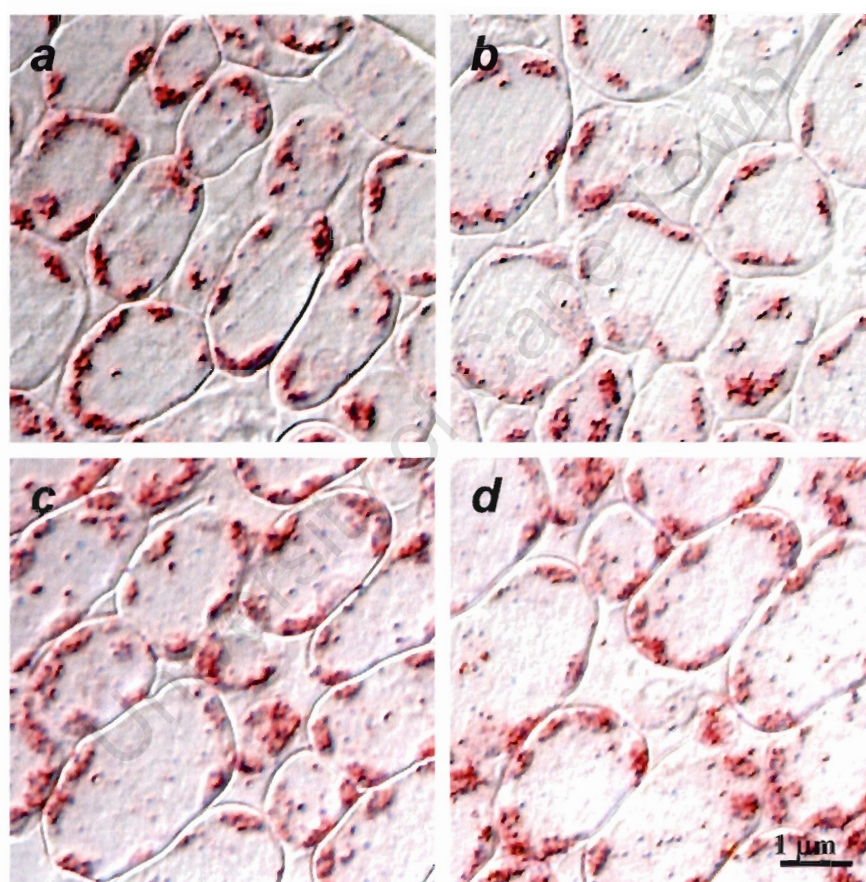


Figure 4.5. Immunolocalization of the cytosolic APX protein in water treated (*a* and *b*) and 100 μ M ABA treated (*c* and *d*) *X. viscosa* leaf tissue using the Fast-Red precipitation technique (Sigma, UK).

No significant expression of XvPer1 was detected in water-treated leaf tissues as shown in Figure 4.6. However, in ABA treated tissues, a clear immunoprecipitation reaction was observed. As well as being clearly associated with the nucleus, substrate

precipitation was observed in the cytoplasm of the ABA treated tissues as compared to the water treated tissues. Some precipitation was also present in the cell wall area, as compared to the water control. This therefore suggests that XvPer1 is located to the nucleus under stress conditions, but is also present in the cytoplasm, and possibly in the cell wall. A significant space can be observed between the cytoplasm and cell wall. This observation is usually present in water-stressed plant tissues. It might be possible that under ABA stress, similar mechanisms as in water-stressed tissues are activated.

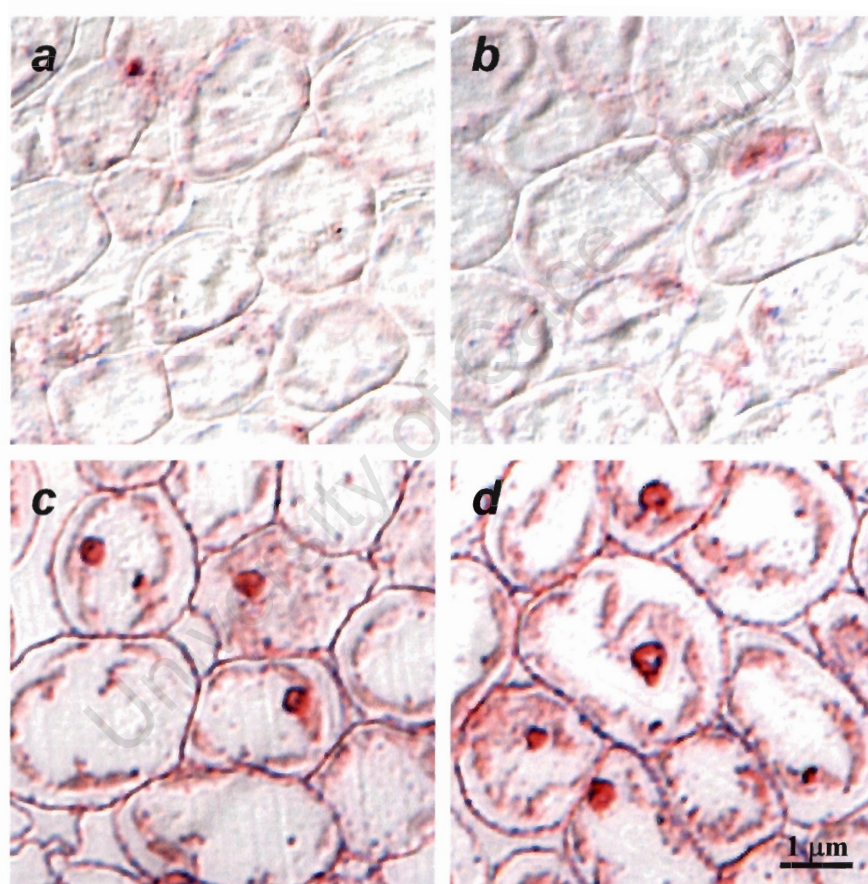


Figure 4.6. Immunolocalization of the *X. viscosa* XvPer1 protein in water treated (*a* and *b*) and 100 μ M ABA treated (*c* and *d*) *X. viscosa* leaf tissue using the Fast-Red precipitation technique (Sigma, UK).

4.4 DISCUSSION

Analysis of the amino acid sequence of XvPer1 revealed that it had a putative bipartite NLS (see section 2.3.1). Two different immunolocalization studies were carried out. Firstly with dehydrated *X. viscosa* leaf tissues, and secondly with ABA treated leaf tissues, to try and elucidate the subcellular localization of XvPer1. Western blot analysis has revealed that XvPer1 is absent in *X. viscosa* vegetative tissues under un-stressed and fully hydrated conditions, but is highly induced under both dehydration stress and upon treatment with the plant stress hormone ABA (see section 3.3.5). So far only a few desiccation-associated proteins have been found localized to the nucleus (RAB17, Goday et al., 1994; TAS14, Godoy et al., 1994; RAB28, Niogret et al., 1996; GP47, Chiantante et al., 1995; PER1, Stacy et al., 1999). The RAB17 protein from maize is ubiquitously distributed in the embryo tissues and is also ABA inducible (Goday et al., 1994). Immunolocalization data has showed that in maize embryos, RAB17 is associated with nuclear particles as well as being present in the cytosol. Goday et al (1994) propose that RAB17 may function by interacting with specific proteins through association with their NLSs during stress conditions, either by sequestering them in the cytoplasm, or functioning as an import/export carrier.

The TAS14 protein from tomato was induced in tomato seedlings upon treatment with ABA, NaCl and mannitol (Godoy et al., 1994). Immunogold electron microscopy has localized TAS14 both to the cytosol and in the nucleus. The maize RAB28 protein level was found to increase under conditions of dehydration, both during late embryogenesis in seed development and in vegetative tissues (Niogret et al., 1996). Immunogold electron microscopy showed accumulation of RAB28 in the nucleolus. However no function has been associated to it thus far. Its Arabidopsis orthologue (Atrab28) is also present in embryo tissues, but is not induced in vegetative tissues by ABA or dehydration (Arenas-Mena et al., 1999). The PER1 1-Cys peroxiredoxin, which is the barley orthologue of XvPer1, was detected in the embryo epidermal cells and in the root and shoot apex (Stacy et al., 1999). The PER1 protein levels are stable up to 11 days post imbibition in dormant barley embryos, but considerably less in non-dormant embryos. The PER1 antiserum was found to react most strongly against

the nucleus of developing embryo and aleurone cells, with highest signal levels in the nucleolus.

However, in mature imbibed dormant seeds, cytosolic levels of the protein are comparable to that in the nucleus. The authors postulate that during active protein translation, the protein is present in the cytoplasm, but is thereafter translocated to the nucleus. Alternatively, or additionally, the PER1 protein might be performing different tasks in the cytoplasm and the nucleus, which is specific to the tissue type. PER1 has been shown to have antioxidant activities in vitro (Stacy et al., 1996). Using a Mixed Function Oxidation assay (MFO), Stacy et al. (1996) showed that purified PER1 could reduce oxidative damage to DNA in vitro. Therefore PER1 has been postulated to protect seed nuclear components against free radical damage during desiccation (Stacy et al., 1999). The same MFO assay was used to illustrate that the Arabidopsis 1-Cys peroxiredoxin (AtPer1) can also protect DNA against damage by ROS, although to a lower extent (Haslekas et al., 2003a). Analysis of AtPer1 in PSORT and PredictNLS Software predicted that the protein is most likely to be found in the cytoplasm. Immunolocalization of AtPer1 in Arabidopsis has not yet been carried out.

Both the immunofluorescence localization and localization using Sigma Fast-Red precipitation showed that XvPer1 is a highly abundant protein under conditions of stress. A low level of protein was detected in the cytoplasm under control conditions which increases significantly under stress. It is clearly localized to the nucleus of *X. viscosa* leaf cells under conditions of dehydration stress (Fig. 4.3) (Mowla et al., 2002) and upon treatment with ABA (Fig. 4.6). This suggests that the putative NLS present in the XvPer1 amino acid sequence is functional. However further studies of the NLS is required to confirm this. Analysis of the Arabidopsis orthologue, AtPer1, using PSORT and PredictNLS software predicted that the most likely localization for the protein is the cytoplasm (Haslekas et al., 2003a). Additionally, a GFP-AtPer1 fusion was localized to the cytoplasm and the nucleus. However, due to similar localization patterns of GFP without AtPer1, which does not contain an NLS, it cannot be excluded that the localization seen was due to diffusion.

A degree of binding of the XvPer1 antibody with components in the cytoplasm is also apparent mostly in ABA-treated *X. viscosa* leaf tissue using the Fast-Red detection system. Unfortunately no preimmune-serum was available to use as a control in this experiment. However, control leaf tissue (sprayed with H₂O instead of ABA), showed no cross-reactivity with cytoplasmic components when probed with XvPer1 antibody. Which leads to the conclusion that the result obtained is genuine. When investigating the immunolocalization of the H3 protein and the APX protein by immunoprecipitation, no colour development was detected in the cell wall area, neither under control conditions nor under stress conditions. Similarly, no such precipitation was observed in water-control tissue probed with XvPer1. However, in ABA treated tissue, a distinct precipitation in the cell wall area was detected in ABA treated leaf tissue probed with XvPer1. This suggests that XvPer1 could be associated with the lipid membrane, although a hydropathy profile of the XvPer1 amino acid sequence has revealed that the protein is not particularly hydrophobic (see section 2.3.1). Alternatively, this could be non-specific binding.

So what are the potential functions of XvPer1? As mentioned earlier, the generation of ROS is an inevitable process in all aerobic organisms, but is over-produced especially under conditions of pathological and physiological stress (Lee et al., 2000). ROS can cause serious damage to biological macromolecules, such as proteins, lipids and nucleic acids, and can potentially even lead to cell death. Various antioxidants are produced to counteract this, including xanthophyll pigments, β -carotenes, glutathione, superoxide dismutase, catalase and ascorbate-dependent peroxidase. Peroxiredoxins have been discovered fairly recently, and are unrelated to any other peroxidase families (Hofmann et al., 2002). The subcellular localization of the XvPer1 1-Cys Prx in the nucleus indicates a role in protecting DNA and the nuclear machinery of transcription. An in vitro assay using the MFO system and purified native recombinant XvPer1 protein is necessary to confirm this theory. XvPer1 has also been localized to the cytoplasm, and possibly to the cell wall. It is possible that during active translation, XvPer1 is found in the cytoplasm, but is thereafter translocated to the nucleus. However, the possibility that XvPer1 performs an active protective function in the cytoplasm as well cannot be excluded. Again, certain activity assays using the purified XvPer1 protein are necessary in order to elucidate any antioxidant

activity of the protein. It is clear that a better understanding of the function of the *X. viscosa* 1-Cys Prx protein and its genetic manipulation is necessary to allow us to determine the usefulness of *XvPer1* in the development of stress-tolerant transgenic plants. Further experiments in the localization of XvPer1 are underway and include the study of the constitutive expression of an XvPer1-gfp (green fluorescent protein) fusion in transgenic *Arabidopsis* plants.

University of Cape Town

CHAPTER FIVE

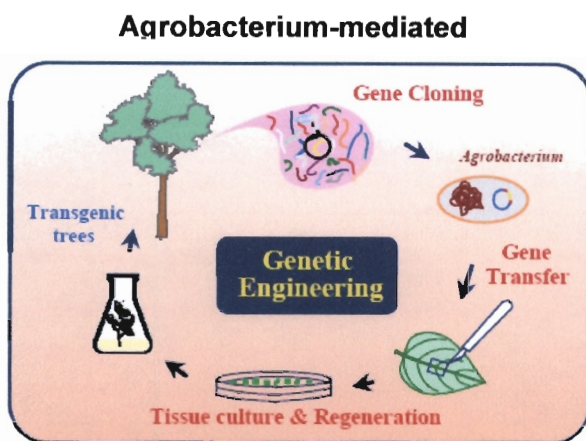
OVER-EXPRESSION OF *XvPer1* in *ZEA MAYS*

SUMMARY

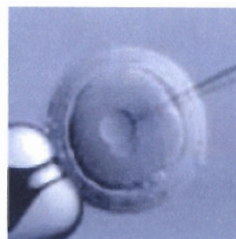
The *XvPer1* cDNA was transformed into the crop plant *Zea mays*. The cDNA was cloned into the plant transformation vector pA53 under the control of the constitutive ubiquitin promoter. The pA53-*XvPer1* recombinant plasmid, together with another plant transformation vector pUbibar35S, were co-bombarded into maize calli. Positive transformants were firstly selected in tissue culture with bialaphos selection. Thereafter, positive transformants were identified by PCR on genomic DNA and western blot analysis. T₁ generation control and transgenic maize seedlings were subjected to high light stress (1000 $\mu\text{molm}^{-2}\text{s}^{-1}$) and their phenotypes assessed. The leaves of control plants curled under the stress and were bleached while transgenic plants did not display such phenotypes. Control plants also grew slower than the transgenic.

5.1 INTRODUCTION

The transgenic approach is a useful tool to investigate gene function. The production and analysis of transformants is one of the most efficient ways in the discovery of gene function. This can be done in two ways: either by silencing the gene of interest, in which case the gene under investigation will have to have a very close orthologue in the model plant being used, or by over-producing the gene of interest in a model plant of study such as *Arabidopsis*. Transgenic plants carrying the gene of interest under the control of a new promoter (constitutive, tissue or cell type specific, inducible) can be ideal experimental material for molecular, biochemical or physiological studies. In addition to using transgenic plants for functional studies, this technology is being used to give plants new traits that benefit agricultural production. The ability to move genes into plants from unrelated plants or other organisms has resulted in significant achievements in biotechnology which would have been impossible with traditional breeding. Three major ways exist for introducing new genes into plants (Fig. 5.1).



Electroporation



Particle bombardment



Figure 5.1. Three major methods used in biotechnology for introducing new genes into plant cells (<http://forest.mtu.edu/faculty/tsai/FW3075/3075-18.pdf>).

After the event of plant transformation, successful integration of the transgene needs to be selected from the background of non-transgenic plants. Antibiotic resistance genes are good selectable markers for dicot plants. Most monocot plants are resistant to common antibiotics and herbicide resistance genes are preferred. However, one of the environmental risks involved is the escape of foreign genes from transgenic crops via sexual reproduction with a non-transgenic variety of the same crop or a sexually compatible relative. This may lead to the pollution of fields with unwanted herbicide resistant plants. Such an incident has been reported by Hall et al. (2000) where herbicide resistance transfer via pollen was observed among *Brassica napus* varieties in a relatively short space of time.

It is serious concern for biotechnologists and one way of dealing with this problem has been to use a “transformation segregation” scheme in which two independent

transformation vectors are co-transformed into the plant. One vector contains the gene of interest while the other contains the herbicide resistance selectable marker. Because the two genes are carried on separate vectors, they are integrated in different locations on the plant genome. This allows for initial selection of transgenic plants by herbicide resistance, but future progenies of the transgenic plants will lead to the segregation of the two genes and hence the transgenic plants only expressing the gene of interest can be selected.

In this chapter cloning of *XvPer1* will be described followed by transformation into *Z. mays* calli using biolistics. Successful transformants will be selected and an assessment of their performance under high light stress compared to control plants will be presented.

5.2 MATERIALS AND METHODS

5.2.1 Cloning of *XvPer1* into pA53

Figure 5.2 shows the map and features of the plant transformation vector pUbi.gfp.nos (pA53) (Shirasu et al., 1999). The plasmid was originally designed to produce green fluorescent protein (gfp) fusions with other proteins of interest for localization and translocation studies in monocot plants. The plasmid is 6230 bp, which is relatively small for plant transformation vectors. Another attractive feature is its MCS which consists of numerous restriction enzyme sites. The expression of *gfp* in the plasmid is driven by the ubiquitin promoter and a *nos* terminator. An ampicillin selectable marker allows for bacterial selection. No selectable marker for selection in plants is present. The *gfp* fragment was removed by restriction analysis using *Bam*HI and *Kpn*I and directionally replaced by the *XvPer1* cDNA coding sequence.

pA53-*XvPer1* was transformed into *E. coli* DH5 α competent cells and selected on agar plates containing ampicillin (100 μ g/ml). Positive transformants were initially checked by colony PCR using a pA53 specific forward primer designed upstream of the MCS (5'-TCGACTCTAGGATCCCCCG-3') and an *XvPer1* specific reverse primer (5'-CATTCACTCAGACGTTTCGTAAAACG-3'). Positive colonies were used for plasmid isolation and the sequence was analysed by ALFexpress automated DNA

sequencer AMV3.0 (Pharmacia Biotech, USA) using the sequencing Fluorescent Labelled Primer cycle sequencing kit (Amersham International).

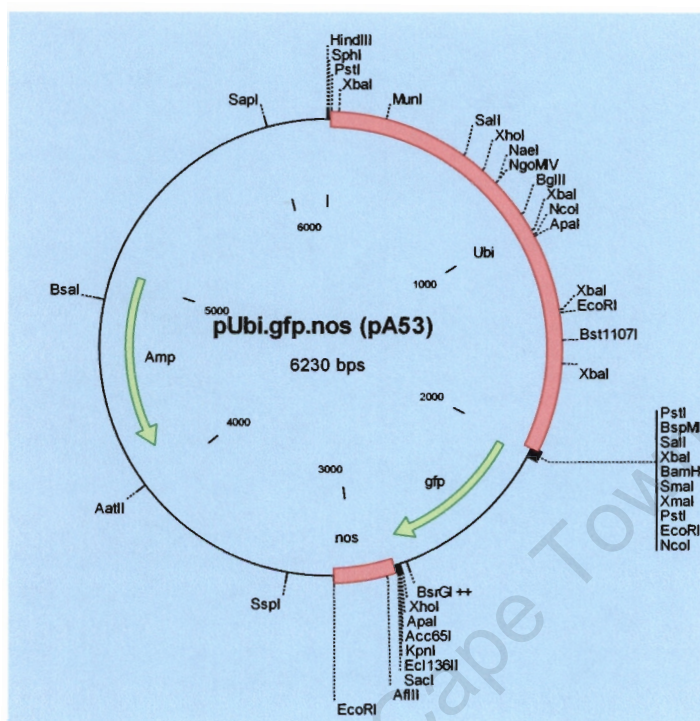


Figure 5.2. Map of pUbi.gfp.nos (pA53) (Shirasu et al., 1999).

Because pA53 does not contain a selectable marker for plants, maize calli were co-bombarded with the vector pUbibar35S (Fig. 5.3) (Toki et al., 1992). This vector was developed from the pActbart35S vector, from which the *bar* gene (coding for bacterial phosphinothricin acetyl transferase linked to a 35S poly-A terminator) was taken, and the pUbicryBnos vector from which the ubiquitin promoter linked to an intron was taken. Those two elements were then cloned into a pGEM3Zf(+) backbone to create pUbibar35S. The latter contains the *bar* gene, which gives resistance to the herbicide bialaphos, constitutively driven by the ubiquitin promoter and controlled by a 35S poly A terminator. The intron enhances the expression of the *bar* gene.

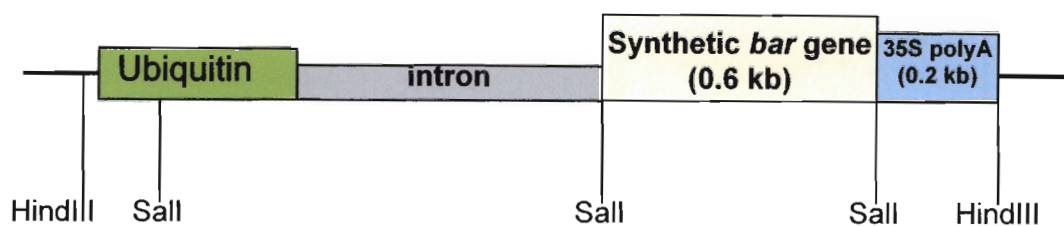


Figure 5.3. Main features of the pUbibar35S vector cloned in a pGEM3Zf(+) backbone (adapted from Toki et al., 1992).

5.2.2 Transformation of *Z. mays* Calli by Microprojectile Bombardment

Calli derived from embryonic HiII maize cells (courtesy of Mr Marc Galatowitsch, University of Minnesota) were bombarded using a PDS-1000/He Biolistic Bombardment Delivery System (Biorad). This system accelerates the gold particles by helium pressure under a vacuum. 0.6 μ M gold particles (Biorad) were mixed with the plasmids pA53-XvPer1 and pUbibar35S in a 1:1 ratio. Bombardment was performed as recommended by the manufacturer and using published data (Klein et al., 1989). Each plate of calli received two shots of gold and were placed in the dark O/N before transferring onto normal MS media (Murashige and Skoog, 1962).

After five days in the dark, the calli were transferred onto MS-regeneration media (MS media with 0.1 mg/ml NAA and 1 mg/ml BA) to encourage shooting with bialaphos selection (Meiji Seiki Kaisha LTD, Japan; 1.5 mg/ml). The plates were kept in the dark for another five days before being moved under filtered light. Regenerated calli were transferred onto MS-rooting media (4,32 g/L MS salts, 10 ml/L MS vitamins, 30 g/L sucrose, pH to 5.8 with 1 M KOH, 8 g/L agar) with bialaphos selection (3.0 mg/ml). Plants with well developed shoots and roots were transferred into soil and placed into a growth room with the following conditions: 16/8 hours day/night cycle, at a temperature of 26-28°C and a light intensity of 700 μ molm⁻²s⁻¹.

5.2.3 Testing for Positive *XvPer1* Transformants

Genomic DNA was extracted from putative transgenic plants using the following protocol adapted from Davis et al. (1980). 500 mg of leaf tissue was ground in 1 ml of extraction buffer (100 mM Tris pH 8.0, 50 mM EDTA pH 8.0, 500 mM NaCl) with 1 μ l β -mercaptoethanol. 70 μ l of 20% SDS was added, mixed thoroughly by vortexing

and the tube incubated at 65°C for 10 minutes. 350 µl of 5 M potassium acetate/acetic acid mixture (60 ml of 5 M potassium acetate, 11.5 ml of acetic acid, made up to 100 ml with water) was added, the tube vortexed and incubated at 0°C for 20 minutes, then centrifuged at maximum speed for 20 minutes. The supernatant was transferred to a new tube and 700 µl of isopropanol added, gently mixed, and incubated at -20°C for 30 minutes. After incubation, the precipitated DNA was collected into a pellet by centrifugation at maximum speed, the pellet was air-dried and redissolved in 50 µl TE buffer (50 mM Tris, 10 mM EDTA) with 3 µl of RNaseA (10 mg/ml). The tube was centrifuged again to remove insoluble debris and the supernatant transferred to a new tube. 5 µl of 3 M sodium acetate and 35 µl of isopropanol was added and the precipitated DNA collected again by centrifugation. The pellet was washed in 80% ethanol, air-dried and redissolved in 100 µl of TE buffer.

Typically, 300-500 ng of DNA was used in a 50 µl PCR reaction. Primers specific to the *XvPer1* cDNA were used in the PCR reaction. PCR reactions were viewed on EtBr-stained 1% TBE gels.

For western blot analysis soluble proteins were isolated from leaf tissue of the putative transgenic plants using the Trizol reagent (Invitrogen, USA) as outlined in section 3.2.3. Equal amounts of protein samples were separated by SDS-PAGE (12%) and transferred onto nitrocellulose membranes. Western blot analysis was performed as described in section 3.2.8.

Positive transgenic plants were crossed with WT maize plants (University of Kwazulu-Natal, Pietermaritzberg; South Africa) to increase the probability of fertilization and T₁ seed production. T₁ seeds were planted and tested for *XvPer1* expression as described above.

5.2.4 High Light Stress

Three maize control seedlings (plants which went through the transformation process but which did not incorporate *XvPer1* into their genome) and three *XvPer1* transgenic seedlings were subjected to a high light stress (1000 µmolm⁻²s⁻¹) two weeks after they had been transferred into soil. All other conditions of the growth room stayed

unchanged. The seedlings remained under high light for a three week period. *Zea mays* plants are generally well able to cope with a light intensity of $1000 \mu\text{molm}^{-2}\text{s}^{-1}$, however this light intensity may be stressful for seedlings, especially those grown in incubators rather than in the field.

5.3 RESULTS

5.3.1 Identification of Transgenic *Z. mays* Over-Expressing *XvPer1*

PCR on genomic DNA identified maize plants which contained the *XvPer1* cDNA incorporated into their genome. The rest of the plants, although resistant to bialaphos, did not carry the *XvPer1* gene. This is possible as the two genes were bombarded into the calli on separate vectors and only the *bar* gene could have integrated into the genome. Those plants were used as controls. Figure 5.4 shows maize calli used for transformation and putative transgenic maize seedlings in soil from which genomic DNA was isolated.

Figure 5.5 represents the PCR results from 15 putative *Z. mays* transgenic plants selected on bialaphos. Wild type DNA was used as a negative control. 13 out of the 15 plants tested here gave a positive band similar to the positive control at ~650 bp.

Western blot analysis revealed that many of the PCR positive maize plants did not produce the *XvPer1* protein. Figure 5.6 is one representation of a western blot analysis result from seven putative transgenic plants. Only one plant showed definite expression of the *XvPer1* protein. A very faint band representing an apparent dimeric form (~60 KDa) of the *XvPer1* protein was present in some of the other plants tested. This band was especially strong in the positive control, which represents 1.5 μg of purified recombinant His-tagged *XvPer1* protein (see section 3.2.6). Although a denaturing SDS-PAGE gel was used, it seemed complete denaturation was not achieved.

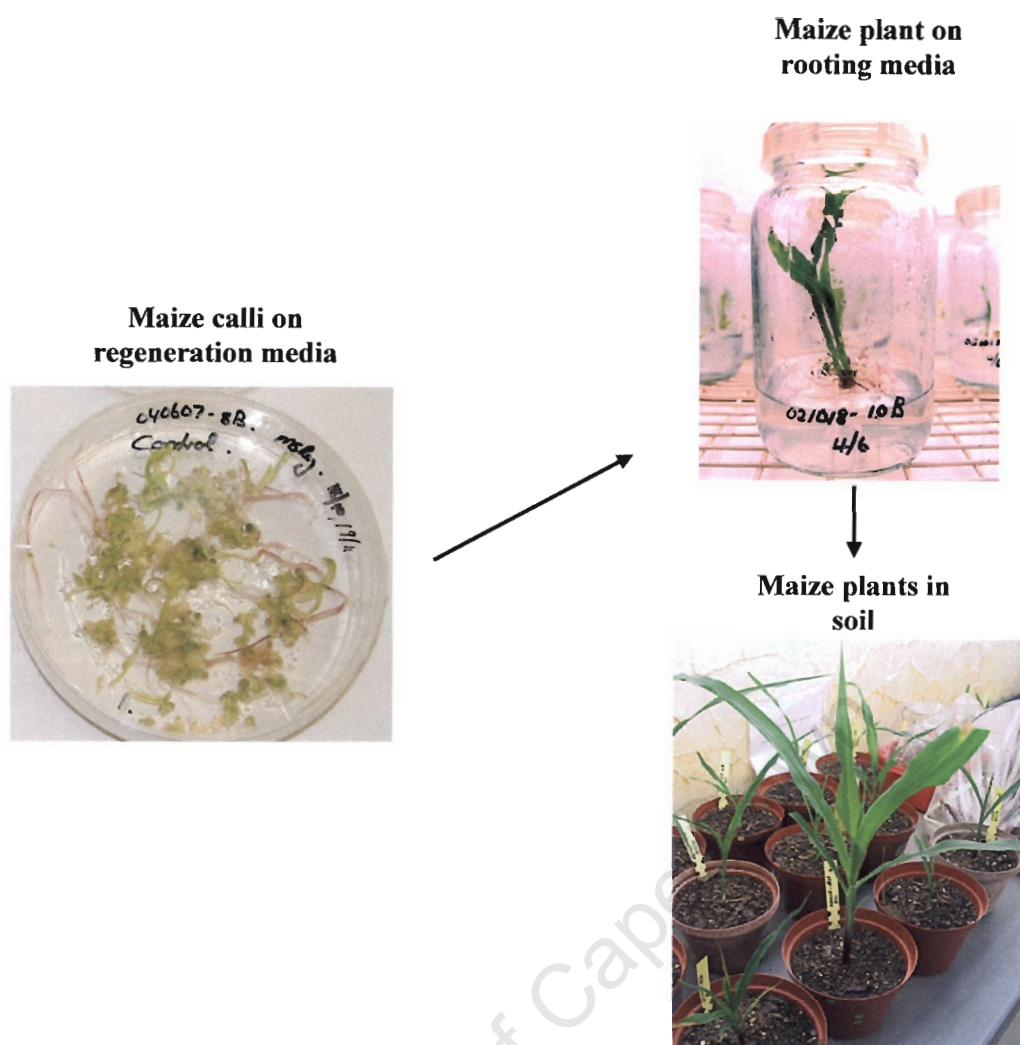


Figure 5.4. *Z. mays* calli developed from immature embryo co-bombarded with pA53-XvPer1 and pUbibar35S on regeneration and rooting media containing bialaphos (3 mg/ml) and transgenic maize seedlings in potted soil.

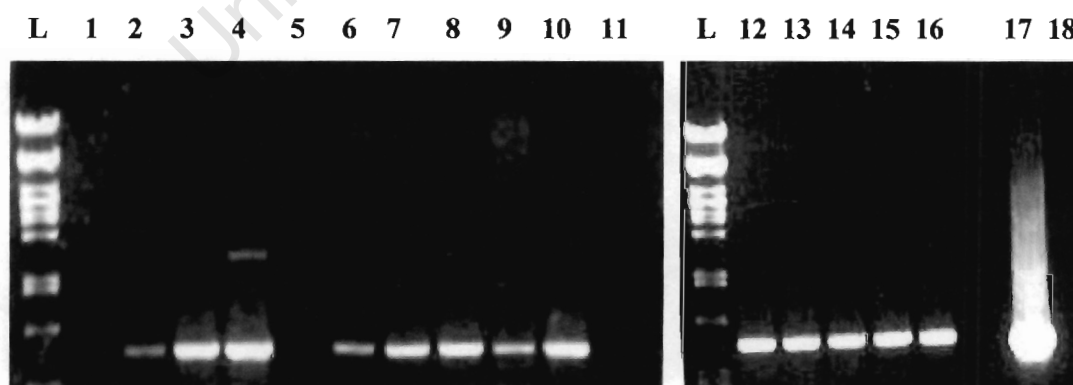


Figure 5.5. Visualization on 1.0% EtBr-stained TBE agarose gel of PCR on genomic DNA of putative *Z. mays* transgenic plants using *XvPer1* specific primers. L: λ -PstI DNA ladder; lane 1: wild type (negative control); lanes 2-16: fifteen independent putative transformant lines (8A1, 8A2, 8A3, 8A4, 13D1, 13C1, 13C2, 4A1, 14A1, 19A3, 19A4, 19A5, 19A6, 19A7,

19A8); lane 17: pA53-XvPer1 plasmid template in PCR reaction (positive control); lane 18: no DNA in PCR reaction (negative control).

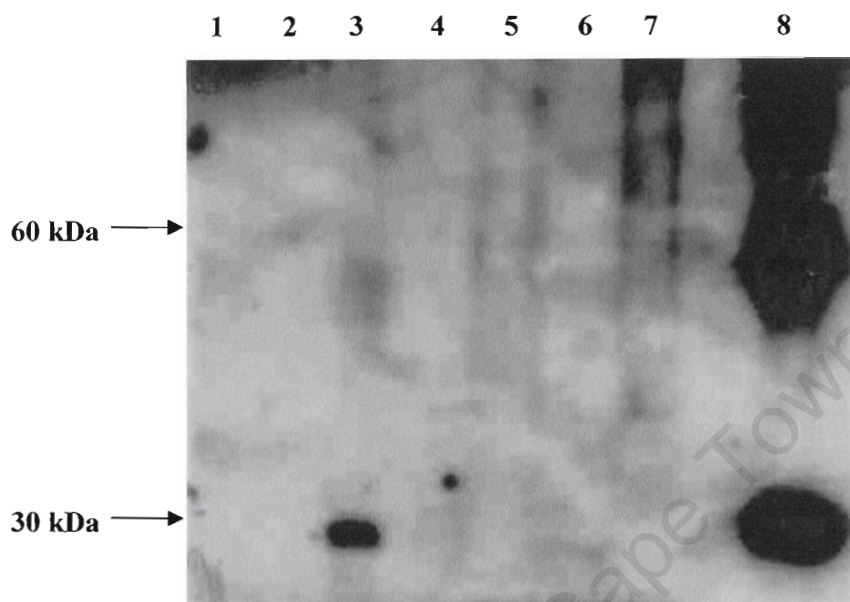


Figure 5.6. Western blot analysis of XvPer1 protein in transgenic *Z. mays*. Total soluble protein was separated by SDS-PAGE and blotted onto nitrocellulose membranes which were incubated with polyclonal anti-XvPer1. Lanes 1-7: putative transgenic lines (16A7, 9D2, 16G1, 13G1, 13E2, 7A2, 7A4); lane 8: pure XvPer1 recombinant protein (positive control).

5.3.2 Transgenic *XvPer1 Z. mays* under High Light Stress

Three maize seedlings over-expressing the *XvPer1* transcript, and three control plants (plants giving negative results of *XvPer1* expression by PCR) were subjected to a high light stress of $1000 \mu\text{molm}^{-2}\text{s}^{-1}$ for a period of three weeks. Before the beginning of the stress, both transgenic and control plants had similar phenotypes (Fig. 5.7). At the end of the stress treatment, control plants were shorter than *XvPer1* transgenics. Additionally, the leaves of control plants had a 'curled' phenotype and a low degree of bleaching was observed. No such observations were made for the transgenic plants. However, this result is preliminary. The differences between the plants will have to be quantified scientifically. This will be done in the future when the experiment is repeated on homozygous transgenic lines.

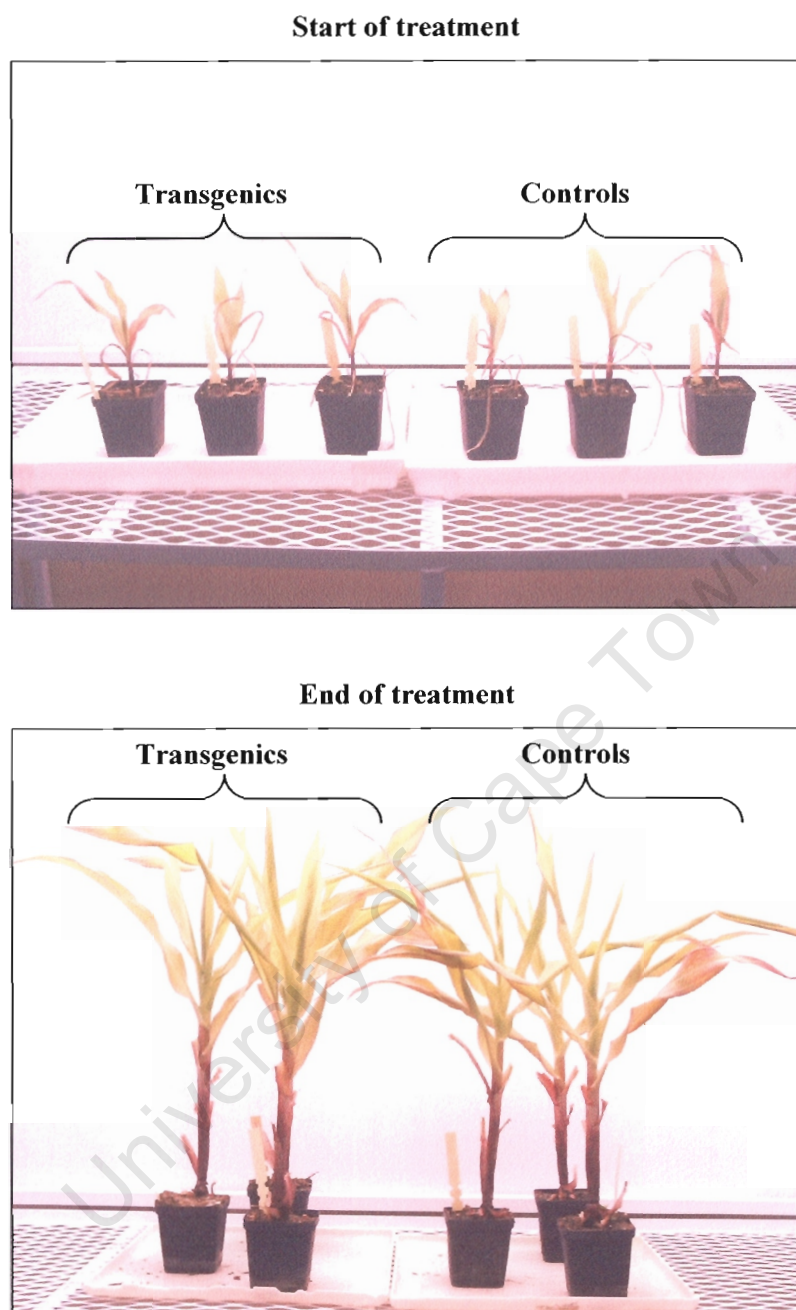


Figure 5.7. The phenotypes of three control maize plants and three *XvPer1* over-expressing transgenic *Z. mays* plants before and after high light stress treatment ($1000 \mu\text{molm}^{-2}\text{s}^{-1}$).

5.4 DISCUSSION

The ability to be stress tolerant is generally known to be a multi-genetic trait (Zhu et al., 1997). A number of genes are responsible for the biochemical and physiological processes involved in enabling a plant to be stress tolerant. Therefore, it is generally recommended that in order to achieve meaningful tolerance to certain abiotic stresses, such as drought and salinity, the expression of several stress-responsive genes, or several genes involved in a pathway leading to stress tolerance, be expressed in a single genotype (Cushman and Bohnert, 2000). For example, betaine is an important osmoprotectant, and is produced in many plants in response to abiotic stresses (Hanson et al., 1998). Recently, a biosynthetic pathway of betaine from glycine, catalyzed by two *N*-methyltransferase enzymes was found. The co-expression of the genes encoding for these two enzymes in *Arabidopsis* resulted in high accumulation of betaine throughout the plants, with improved seed yields under stress conditions (Waditee et al., 2004). Similarly, the over-expression of multiple dehydrin genes in *Arabidopsis* resulted in improved survival of the plants when exposed to freezing stress as compared to control plants (Puhakainen et al., 2004).

However, the expression of a single gene has also shown stress tolerance improvement in plants. Previous studies have shown that cytoplasmic Ca^{2+} levels in plant cells increase rapidly in response to multiple stress stimuli (Sander et al., 1999). Saijo et al (2000) demonstrated that the over-expression in rice of a single stress inducible gene encoding a Ca^{2+} -dependent protein kinase enhanced the induction of other stress responsive genes and improved cold and drought tolerance in the plants.

By over-expressing a single stress-inducible gene from the resurrection plant *X. viscosa* into maize, it is hoped that firstly, the analysis of the transgenic plants will help to elucidate the function of the protein in stress tolerance. Secondly, that its over-expression will confer a degree of abiotic stress tolerance to the transformed plants. To date, very little has been reported on the over-expression of stress-inducible genes from resurrection plants in heterologous plant systems. Three cDNAs representing three stress-inducible genes from *C. plantagenium* were constitutively expressed simultaneously in tobacco plants (Iturriaga et al., 1992). No changes in phenotype, growth habit or photosynthetic parameters were observed and no increase in drought

tolerance as measured by ion-leakage tests was observed. But more recently, a stress-inducible gene from *X. viscosa* encoding a protein which is an orthologue of WCOR413, a low-temperature regulated highly hydrophobic protein from wheat, was over-expressed in *Arabidopsis* (Garwe et al., unpublished). Transgenic plants showed improved tolerance to osmotic, salt, heat and drought stresses. Tobacco plants over-expressing the same gene had higher fresh and dry weights than untransformed control plants. When exposed to water-deficit stress, the transgenic plants had greater membrane permeability as demonstrated by electrolyte-leakage tests.

Transformation of *XvPer1* into maize using particle co-bombardment resulted in a low number of successful transformants. Out of three independent transformation events, using approximately 30 freshly generated calli each time, only 1% of transformants were found to express the protein. The constitutive over-expression of the *XvPer1* protein did not seem to affect growth as, under normal growth conditions, the transgenic plants had growth patterns similar to control plants. When T₁ generation plants were subjected to drought stress, no difference could be observed between transgenics and control plants (data not shown). However, under high light stress, *XvPer1* transgenic maize displayed a physiological advantage over control plants. After 21 days exposure to the stress, transgenic plants were taller than control plants. Furthermore, the leaves of control plants curled and were bleached, while transgenic plants displayed none of these phenotypes.

This assessment of the maize *XvPer1* transgenic plants is preliminary but indicated that the over-expression of *XvPer1* in the maize crop plant gave the latter a growth advantage under conditions of high light stress. Since the data proved to be promising, further tests will be carried out but cannot be included in this dissertation due to time constraints. Homozygous *XvPer1* over-expressor plants will be obtained by self pollinating the transgenic T₁ generation plants and their progenies. By Southern blot analysis, the number of *XvPer1* insertions in the genome will be determined and the level of expression the *XvPer1* protein will be determined by western blot analysis. Thereafter, a more thorough characterization of the *XvPer1* maize transgenics will be carried out by subjecting the plants to a range of abiotic stresses.

CHAPTER SIX

ANALYSIS OF *AC3*, A LEA-LIKE PROTEIN FROM ARABIDOPSIS

SUMMARY

The Arabidopsis *AC3* cDNA (gene annotation AT4g02380, cDNA annotation AY054147) coding for a LEA-like group 3 protein was chosen because of its ability to confer H₂O₂ tolerance to baker's yeast. Sequence analysis of the *AC3* cDNA revealed that it represents a full-length cDNA of 294 bp with a deduced amino acid sequence encoding a protein of 97 amino acid residues, with a molecular weight of 10.3 kDa. Four possible N-myristoylation sites and one protein kinase C phosphorylation site were identified and a topology search revealed a chloroplast signal peptide. A hydropathy plot showed that the protein is mostly hydrophilic. *AC3* shares 64% similarity with its tomato orthologue, 63% with the potato orthologue, 58% with the tobacco orthologue and 53% with both the *Ammopiptanthus mongolicus* and the mung bean orthologue. Unlike most LEA proteins, *AC3* is not highly expressed in Arabidopsis seeds under control conditions, but high expression was detected in roots. No *AC3* expression was found in stems and leaves while some expression was present in flowers and immature siliques. The *AC3* steady-state mRNA transcript level was found to increase in response to dehydration, with highest expression at 12% Soil Water Content (SWC). *AC3* expression was also induced by the stress hormone ABA (100 µM) and by exogenous application of the pro-oxidants diamide (1 mM), H₂O₂ (10 mM), menadione (50 µM) and paraquat (100 µM). Under dehydration stress, the *AC3* steady-state mRNA transcript levels were highly induced in the ABA insensitive mutant *abil-1* and the ABA deficient mutant *aba1-1*.

6.1 INTRODUCTION

The subject of late embryogenesis abundant (LEA) proteins has been briefly discussed earlier in chapter one (section 1.4.1). Here, LEAs will be discussed in further detail and the role of one particular LEA protein in stress tolerance will be assessed. LEA proteins were first reported 20 years ago in cotton and wheat, but to date their exact function remains obscure (Wise and Tunnacliffe, 2004). LEA proteins

are mostly hydrophilic proteins and based on expression patterns and sequences, at least six different groups of LEA proteins have been defined (Table 6.1) (Cumming, 1999).

Table 6.1. LEA protein groups (Wise and Tunnacliffe, 2004)

LEA group	Sequence motif	LEA superfamily	Consensus POPP ^a
1a	GGQTRREQLGEEGYSQMGRK	4	+E, +G, +EG, +GE, +GG, +KG, +QE, +RK, +GGE, +KGG
1b		6	+E, +G, +DE, +EG, +ES, +GG, +GQ, +RE, +RK, +ARE, +DES, +REG
2a	DEYGNP (Y domain)	1	+G, -L, +EK, +GG, +GT, +EKL, +IKE, +KEK, +KIK, +KKG, +KLP, +LPG
2a	EEKK (K domain)	10	-F, +G, -I, +AG, +EK, +GG, +GQ, +KE, +SS, +EKL, +GAG, +IKE, +KEK, +KLP, +LPG, +SSS
2b	S _n (S segment)	3	-F, -I, -L, -R, -W, +DK, +EK, +KK, +KL, +LP, +TH, +EKK, +KEK, +KLP, +LPG
3a	TAQAAKEKAGE	2	+A, -C, +E, -F, -I, +K, -L, -P, +AE, +AK, +EK, +ET, +GE, +GK, +KE, +AAE, +AKD, +EKA
3b		5	+A, -I, +K, -L, -P, +Q, +T, -V, +AA, +AQ, +EK, +KE, +KT, +QA, +QQ, +QS, +QT, +TQ, +AAK, +AQA, +EKT, +QAA, +TQQ
6	?	7	+A, -F, -L, +AA, +AE, +MQ, +QS, +VA, +AAA, +GVA, +QSA, +SAA

^aA consensus POPP (Protein or Oligonucleotide Probability Profile) is shown for each superfamily, where '+' indicates significant over-expression of a peptide, and '-' indicates significant under-expression. Some minor superfamilies are not shown. The sequences of proteins generally considered to be Group 4 are known (Wise, 2003) to redistribute consistently to Group 2 (PM1_SOYBN, O24442 and LE13_GOSHI) and Group 3 (LE11_HELAN and LE25_LYCES). The two Group 5 proteins, LE29_GOSHI and Q93Y63 are found among the Group 3 LEA proteins. Notice that there is considerable overlap in the consensus POPPs across subgroups, so further work is needed to confirm the biological significance of this refinement.

As their names suggest, they are highly abundant during late embryo development (up to 4% of total cellular protein) (Wise and Tunnacliffe, 2004). However, many LEA proteins are also induced by cold, osmotic stress and exogenous application of ABA (Welin et al., 1994). Various functions have been associated with LEA proteins in their role in desiccation tolerance. They have been proposed to protect cellular structures from the effect of water loss by acting as a hydration buffer, by sequestering ions, by direct protection of other proteins or membranes, or by renaturing unfolded proteins (Cumming, 1999). However, those are supported by relatively little evidence, although their effects on stress tolerance seem apparent. Tomato, wheat and barley LEA proteins have been shown to improve resistance to osmotic or freezing stresses in yeast (Wise and Tunnacliffe, 2004). Additionally, a barley LEA protein improved tolerance to water deficit in rice (Xu et al., 1996) and wheat (Sivamani et al., 2000). More recently, a cDNA coding for a hydrophobic LEA-like protein, *CaLEA6*, was isolated by differential screening from PEG-treated hot pepper leaves (Kim et al., 2005). The protein, predicted to belong to group 6 LEAs, is highly hydrophobic but cytoplasmic. The gene transcript was found to accumulate rapidly when plants were treated with PEG, ABA and NaCl. Furthermore,

tobacco plants over-expressing *CaLEA6* showed enhanced tolerance to dehydration and salinity stresses. Another LEA protein, GmPM16, isolated from soybean cotyledons, was found to have an unusual amino acid distribution along the protein (Shih et al., 2004). Circular dichroism (CD) analysis and Fourier transfer infrared (FTIR) spectroscopy showed that in solution, the protein was highly unordered, with only partial α -helical structures. However, in the presence of SDS and in a dry state, the protein exhibited a conformation of abundant α -helical structures. It also interacts with sugar and forms tightly glassy matrixes in the dry state. It is therefore postulated to play a role in reducing cellular damage during drying by changing protein conformation and forming tight cellular glasses.

Bioinformatics have been used to try and shed some more light on the functions of LEA proteins. Conventional tools such as the BLAST algorithm can show only limited relatedness between LEA proteins and other polypeptides. This is because LEA proteins have sequences of low complexity (peptides whose sequences display significant regularities) (Wootton, 1994).

Table 6.2. Key words and phrases associated with each LEA protein group and super family^a (Wise and Tunnacliffe, 2004).

LEA group	LEA superfamily	Key words and phrases	Possible function(s)
1a	4	Histone H4, chromosomal protein, nuclear protein, methanofuran, DNA binding	DNA binding nuclear protein
1b	6	dsRNA binding, DNA gyrase, (DNA) breakage, CLP, ATP binding	Nucleic acid unwinding or nucleic acid repair Molecular chaperone
2a	1	(DNA) break, ATP binding, DNA topoisomerase, protein biosynthesis, topoisomerase, repair	DNA unwinding or repair
2a	10	Nuclear protein, DNA binding, transcription regulation, intermediate filament, keratin, chaperone, homeobox, coiled coil, HMG box domain, cytoskeletal	DNA-binding nuclear protein; regulation of transcription? Cytoskeleton
2b	3	Coiled coil, nuclear protein, caldesmon, histone H1, chaperone, tropomyosin, filament, (DNA) break, DNA topoisomerase	DNA unwinding or repair Cytoskeleton Ca ²⁺ binding Molecular chaperone
3a	2	Chaperone, coiled coil, tropomyosin, stress, filament, phosphorylation, caldesmon, elongation factor, neurofilament, actin binding, cytoskeleton, rotamase	Molecular chaperone Cytoskeleton Ca ²⁺ binding
3b	5	Coiled coil, histone H1, filament, nuclear protein, neurofilament, antigenic, flagella, HAMP domain, synuclein, peptidoglycan anchor, DNA binding, hsp70	Chromatin associated nuclear protein Filament Kinase or phosphatase?
6	7	GroEL protein, nuclear protein, histone H1, chaperonin, DNA binding, HAMP domain, synuclein, transcription regulation	Molecular chaperone Chromatin associated nuclear protein; transcription factor?

Abbreviations: dsRNA, double-stranded RNA; HAMP, the group of histidine kinases, adenylyl cyclases, methyl binding proteins and phosphatases (see InterPro entry IPR003660); HMG, high mobility group (a DNA binding domain); LEA, late embryogenesis abundant proteins. ^aThe list of protein identifiers was generated by a search of a POPP (Protein or Oligonucleotide Probability Profile) version of SwissProt, with each consensus POPP as the query, and where then submitted to the Protein Annotators' Assistant (after deleting any LEA proteins). The most significant key words and phrases are listed here, together with some of the functional themes that emerge. CLP is an ATP-dependent plant chaperone.

To address this problem, a new computational method called POPP (Protein or Oligonucleotide Probability Profile) has been developed (Wise, 2002). This programme allows proteins to be compared based on their peptide composition, rather than similarities in their sequences. Proteins with similar functions are likely to have the same short polypeptides being over- or under- represented. POPP confirms that LEA proteins are mainly hydrophilic as charged and/or polar residues feature strongly (Wise and Tunnacliffe, 2004). POPP was also used to group the LEA proteins into families and super families (SFs) (Table 6.2). This grouping might provide more insight into their function. Group 2 LEAs appear to split into two subgroups. Subgroups can also be identified for groups 1 and 3. Under the POPP analysis, groups 4 and 5 disappear entirely and redistribute into LEA groups 2 and 3. The most interesting approach of using POPP lies in its ability to investigate a database of POPPs for proteins of known function with similar peptide profiles to query proteins of unknown function. Possible functions of LEA proteins are listed in Table 6.2. Of course, these are hypothetical functions, but it provides scope for future experimental explorations.

LEA proteins are typically unstructured and are often described as being ‘natively unfolded’ or ‘intrinsically disordered’ proteins (Dunker et al., 2001). However, lack of structure does not mean lack of function as folding can occur on binding to biological target molecules. It is possible that LEA proteins might function in the unstructured state, especially if they function as a hydration buffer, ion sink, or water replacement molecule. Another possibility is that folding of the LEA proteins is induced upon the onset of the abiotic stress (Wise and Tunnacliffe, 2004). Recent evidence, using infrared spectroscopic analysis, showed that a conformational shift occurs upon dehydration in the group 3a (SF2) LEA protein (AavLEA1) from the nematode *Aphelenchus avenae* (Goyal et al., 2003). This observation is highly unusual as protein dehydration is more often associated with loss of structure.

The LEA-like protein under investigation here, AC3 (gene annotation AT4g02380, cDNA annotation AY054147), belongs to the group 3 LEAs. AC3 was cloned by complementation of a yeast mutant with an Arabidopsis cDNA library (Theodoulou et al., unpublished data). A *Δyap1* deletion strain of *S. cerevisiae* was transformed with an Arabidopsis cDNA library constructed in the yeast expression vector, pFL61

(Minet et al., 1992; the cDNA library was kindly provided by Prof. F. Lacroute, Centre de Génétique Moléculaire, CNRS, 91190 Gif sur Yvette, France). Transformants selected on minimal medium lacking uracil (SD-U) were collected by washing in SD-U and re-plated on SD-U containing 0.38 mM H₂O₂. Plasmids were isolated from putative positive clones and used to retransform the mutant in order to confirm complementation. The inserts of plasmids which conferred H₂O₂ tolerance in this secondary screen were then sequenced in their entirety. The sequence of *AC3* has been submitted to GenBank, accession number AY879295. Expression of *AC3* conferred H₂O₂ tolerance to baker's yeast. Because of its possible role in oxidative stress tolerance, *AC3* was investigated further.

6.2 MATERIALS AND METHODS

6.2.1 Sequence Analysis of *AC3*

The nucleotide sequence of the *AC3* cDNA was determined on both forward and reverse strands using Dye Terminator sequencing (Oxford University, UK). The inferred amino acid sequence of *AC3* was obtained by translation of the cDNA sequence using the DNAMAN software (Version 3.0, 1997). The amino acid sequence of *AC3* was used in the BLAST programme of the National Centre for Biotechnology Information (Altschul et al., 1990) to search databases for sequence similarities. Amino acid sequence comparisons were done with the CLUSTAL programme of DNAMAN. The Bioinformatics and Biological Computing Unit, Weizmann Institute of Science, (Israel) was used to plot the hydrophilicity/hydrophobicity of *AC3*. The ScanProsite tool provided by ExPASy (www.expasy.org) was used to scan *AC3* for conserved motifs. A topology search was performed using iPSORT Prediction (www.psort.org).

6.2.2 Plant Material and Growth Conditions

Arabidopsis wild type (WT) Columbia seeds (Col 0) were obtained from the Boyce Thompson Institute for Plant Research, New York. *Arabidopsis* seeds were germinated on water saturated prescription compost (prepared by Petersfield Products) that contained chloronicotinyl (Intercept; 280g / m³) and germinated in a controlled environment cabinet that maintained a temperature of 25°C, a relative humidity of 70 %, a day length of 10 hours with a light intensity of 250 µmolm⁻²s⁻¹.

Ten days after sowing, the seedlings were transplanted into fresh compost and grown under the same conditions. For the investigation of *AC3* steady-state mRNA expression in different parts of *Arabidopsis* under control conditions, plants were grown as above for harvesting of material from leaves, stems, flowers, immature siliques and mature seeds. Immature siliques corresponded to 48 hours old siliques (~5-7 mm long). For root tissue harvest, *Arabidopsis* seeds were sterilized in the following manner. Seeds were suspended in 80% ethanol with 0.1% Triton X-100 for 10 minutes with shaking, followed by 30% sodium hypochlorite with 0.1% Triton X-100 for 10 minutes with shaking, followed by 3x wash with sterile distilled water. The sterile seeds were sown in a sterile hydroponic system using half-strength MS liquid media (Murashige and Skoog, 1962). After 3 weeks, roots were washed in sterile water and harvested. For signalling pathway investigation, *abal-1* and *abil-1* (NASC, UK) mutant *Arabidopsis* seeds of the Landsberg (Ler) background, as well as *Arabidopsis* WT (Ler; NASC, UK), were sterilized as above. The seeds were sown on full-strength MS solid media which was kept at 4°C for 48 hours before placing in a growth chamber maintained at 22°C, a relative humidity of 50%, a day length of 16 hours and light intensity of 250 $\mu\text{mol m}^{-2}\text{s}^{-1}$. After 10 days, the seedlings were transferred onto water saturated prescription compost (prepared by Petersfield Products) that contained chloronicotinyl (Intercept; 280g / m³) and grown in a controlled environment cabinet with conditions as described above.

6.2.3 Plant Stress Treatments

All plant treatments were performed in a controlled environment cabinet with conditions as outlined above unless otherwise stated and were performed on whole plants. Leaf samples were taken by cutting off whole leaves (3-5 leaves; mid-age), immediately wrapping in aluminium foil, flash-freezing in liquid nitrogen and subsequently storing at -80°C until nucleic acid and protein isolation. For the dehydration treatment, soil water content (SWC) was measured by using the following formula: $[(Fw-Dw)/ Fw] \times 100$. Fw is fresh weight and Dw is dry weight (in 80°C oven O/N). The plants were re-watered after the dehydration stress treatment in order to endure that the stress imposed did not kill the plants. All plants used recovered 24 hours after re-watering.

Dehydration

Dehydration stress was carried out on 5-week old plants by not watering the soil for a period of ± 15 days until the SWC was below 20% and the plants looked completely dehydrated.

ABA treatment

The aerial parts of 5-week old whole plants were sprayed with a 100 μM solution of ABA. Sampling was at times 0, 2, 4, 6, 12 and 24 hours after start of treatment.

Pro-oxidant stresses

Five different pro-oxidants were used: 100 μM paraquat (methyl viologen), 50 μM menadione, 10 mM H_2O_2 , 1 mM diamide and 0.5 mM ter-butylhydroperoxide (t-BOOH). All reagents were prepared in solution and sprayed on the aerial parts of whole 4-week old plants. Leaves were harvested 0, 12 and 24 hours after start of treatment. A water control was included.

6.2.4 Measurement of Steady State CO_2 exchange

Gas exchange measurements

Gas exchange measurements were performed on attached leaves using an automated multichamber open-circuit gas exchange system. The system comprises an infrared gas analyser (IRGA; WA-225-MK3, ADC, Hoddesdon, Hertfordshire, UK), a gas analyser (WA-161 2K, ADC, Hoddesdon, UK) and a gas handling (WA-357-MK3, ADC, Hoddesdon, UK). The O_2 concentration in the gas phase was measured with a gas analyser (series 80, Ox-An Systems, Huddersfield, UK). A gas blender (Signal Instruments Co., Croydon, UK) regulated the CO_2 and O_2 compositions of the air entering the leaf chambers. The flow rate was kept at 9 $\text{cm}^3/\text{second}$ by mass flow meter and controllers (Bronkhorst HI-Tech B.V., Holland). Temperature and relative humidity of the air in the chamber was regulated by bubbling the gas stream through water at 20°C (optimal experimental conditions), and then through a condenser set to the required dew point. Chamber temperature was measured with thermocouples. A capacitance humidity sensor (Vaisala, Helsinki, Finland) was used to measure water content of the air before and after passing through the leaf chamber. White light was

supplied by metal-halide lamps (Wotan, Phillips, Holland) and was measured with selenium sensor (Megatron, London, UK).

Photosynthetic measurements

The CO₂ assimilation rate was measured as follows. Leaves were allowed to attain steady state photosynthesis for 20-30 minutes at constant conditions prior to measurement. Measurements were taken over a 1 hour period. Leaf chamber CO₂ concentrations (C_a) were maintained at 350 ± 10 µmol/mol and O₂ at 210 mmol/mol. Relative humidity in the leaf chamber was 50-60% and the light intensity was set at 800 ± 35 µmolm⁻²s⁻¹. The temperature of the leaf chambers was set at 20 ± 0.5°C. Steady-state CO₂ assimilation of leaves was taken to assess the average steady-state assimilation for the respective plant line.

The apparent quantum efficiency (AQE) was determined from the light response curves for photosynthesis measured from 0 to 1500 ± 100 µmolm⁻²s⁻¹. Irradiance was varied using neutral density filters (Lee Filters, A.C. Lighting, Bucks, UK). Leaf chamber CO₂ concentrations were maintained at 350 ± 10 µmol/mol. Linear regression of the initial slope of the hyperbolic light response curves allows AQE calculation at points approximately 50 µmolm⁻²s⁻¹.

6.2.5 Total RNA Isolation

Total RNA was isolated from all tissues using the Trizol reagent (Life Technologies, Germany). 100 mg of tissue was ground in liquid nitrogen until a fine powder is formed. 2 ml of Trizol was added and ground into a frozen paste. The sample was ground continuously until it had completely thawed. After 5 minutes at RT the sample was centrifuged at 12000xg for 15 minutes at 4°C. The supernatant was transferred to a new sterile microcentrifuge tube and mixed with 0.2 volume of chloroform for 15 seconds. After 5 minutes at RT, the sample was centrifuged again at 8000xg for 10 minutes at 4°C. The upper phase of the partitioned sample was transferred carefully, without disturbing the interface, into a fresh tube. An equal volume of chloroform was added to each sample, mixed as before (15 seconds) and centrifuged at 8000xg for 10 minutes at 4°C. The top aqueous phase was transferred carefully to a fresh tube. Isopropanol was added (0.5 volume) to the sample, gently shaken and left to incubate

at RT for 30 minutes. The precipitated RNA was sedimented by centrifugation at 12000xg for 10 minutes at 4°C. The pellet was washed with 75% ethanol and then dried in a desiccator. The RNA was resuspended in DEPC-treated water and stored at -80°C until required.

6.2.6 Northern Blot Analysis

For northern blot analysis, 10 µg of RNA samples were electrophoresed. The samples were prepared as follows: 10 µg of total RNA sample was mixed with 4 µl of 10xMEN (83.3 g MOPS, 8.2 g NaOAc, 7.45 g EDTA), 6 µl formaldehyde and 20 µl formamide. The samples were heated at 56°C for 30 minutes to denature the RNA. Lastly, 5 µl of EtBr-loading buffer was added [100 µl of DNA loading buffer (50% glycerol, 1 mM EDTA and bromophenol blue for colour) mixed with 8 µl of EtBr stock solution]. The RNA mixture was electrophoresed in a formaldehyde 1.2% agarose gel (1xMEN with 15% formaldehyde) using 1xMEN as running buffer. After electrophoresis, the RNA was transferred onto nylon membrane (Hybond-X+; Amersham Pharmacia Biotech) using 20xSSC (173 g/L NaCl and 88 g/L sodium citrate in DEPC water, pH to 7.0 with NaOH) and after O/N transfer the membrane was UV cross-linked using a stratalinker (Stratagene, USA). To estimate whether equal amounts were loaded, the RNA was visualised by EtBr-staining. Prehybridization was carried out at 65°C in prehybridization buffer (0.5 M NaH₂PO₄, 1 mM EDTA, 7% SDS, 1% BSA) for a minimum of 2 hours. The AC3 probe was labelled with ³²P-dCTP using a random primed labelling kit according to the manufacturer's instructions (Invitrogen, UK). After prehybridization, the radiolabelled probe was denatured (placed in a boiling water bath for 10 minutes and then cooled on ice) and was added to the membrane. Hybridization was carried out at 60°C for 16 hours with gentle shaking. The membrane was washed twice at 60°C for 10 minutes in wash buffer B (0.1% SDS, 0.5xSSC) and autoradiographed at -70°C onto high-performance-autoradiography film (Amersham Pharmacia Biotech, USA). After 5 days exposure, the film was developed manually using developer and fixer reagents (Sigma, UK) according to manufacturer's instructions.

6.2.7 RT-PCR

To investigate the distribution of the *AC3* in different parts of Arabidopsis, cDNA was generated from RNA isolated from stem, leaves, flowers, immature siliques and mature dry seeds and imbibed seeds respectively. Firstly, the RNA sample was treated for genomic DNA contamination in the following way. 2 µg of RNA was mixed with 2 µl of 10xDNaseI reaction buffer, 2 µl of DNaseI Amplification grade (Invitrogen, UK), and made up to 20 µl with DEPC water. The reaction was stopped with 2 µl of 25 mM EDTA after incubation for 15 minutes at RT. After heating the sample for 10 minutes at 65°C, first-strand cDNA synthesis was performed using Superscript II (Invitrogen, UK). The following components were added to a nuclease-free microfuge tube: 2 µl oligo dT (500 µg/ml), 2 µg DNA-free RNA (previous reaction mixture) and 0.5 µl of 25 µM dNTP mix. This mixture was heated to 65°C for 2 minutes and then placed on ice. To the same tube, the following was added: 8 µl of 5xfirst strand buffer and 4 µl of 100 µM DTT. The contents were mixed gently and the tube incubated at 42°C for 2 minutes. 2 µl (200 units) of Superscript was added, gently mixed and the tube incubated at 42°C for 50 minutes. The reaction was inactivated by heating at 70°C for 10 minutes. 1 µl of this cDNA was then used for subsequent PCR reactions using *AC3* specific primers. Forward *AC3* primer composition: 5'-GCGGATCCACTTACTTCGAAA-3', with a BamHI restriction enzyme site incorporated for cloning purposes (underlined). Reverse *AC3* primer composition: 5'-CCTCTAGACTCCTCTTAAAGAC-3', with an XbaI restriction enzyme site incorporated for cloning purposes (underlined). The primers were designed to amplify the complete *AC3* ORF, with the forward primer complementing the sequence just prior to the start of the ATG, and the reverse primer complementing the sequence immediately after the stop codon. PCR thermocycle conditions were as follows: an initial denaturation at 94°C for 3 minutes followed by 35 cycles of denaturation at 94°C for 45 seconds, an annealing temperature of 52°C for 30 seconds and an extension time of 45 seconds at 72°C. The final extension was for 3 minutes at 72°C. As a control to check the integrity of the cDNA generated, a 500bp fragment of the actin transcript (At5g09810) was amplified in all cDNA samples. Actin forward primer composition: 5'-GAGAAGATGACTCAGATC-3'; reverse primer composition: 5'-ATCCTTCCTGATATCGAC-3'. PCR thermocycle conditions were the same as above.

6.3 RESULTS

6.3.1 Sequence Analysis of AC3

AC3 has a nucleotide sequence of 294 bp, representing a full-length cDNA (Fig. 6.1). The cDNA has an open reading frame representing a deduced amino acid sequence encoding a protein of 97 amino acid residues, with a molecular weight of 10.3 kDa and a predicted pI of 10.4 at pH 7. A scan tool suggests one protein kinase C phosphorylation site at position 60-62 (Fig. 6.1). A topology search revealed that AC3 has a chloroplast signal peptide (Fig. 6.1). A hydropathy plot showed that the protein is mostly hydrophilic, as expected of a LEA protein (Fig 6.2). The deduced amino acid sequence of AC3 exhibited a considerable amount of similarity with other plant LEA proteins belonging to group 3 (Fig. 6.3). AC3 shares 64% similarity with its orthologue from tomato (*Lycopersicon esculentum*, TO7161; (Lyoyd et al., unpublished), 63% with the potato orthologue (*Solanum demissum*, CAA66948; Jackson et al., 1997), 58% with the tobacco orthologue (*N. tabacum*, AAC06242; Reinhoud et al., unpublished) and 53% with both the *Ammopiptanthus mongolicus* orthologue (AAW31666; Liu et al., unpublished) and the Mung Bean orthologue (*Vigna radiata*, T10900; Yamatoko, unpublished).

```
1   ATGGCTCGTTCTATCTCTAACGTTAAGATCGTATCTGCTTTTCGTCTCTCGTGAACCTCTCC
1   M A R S I S N V K I V S A F V S R E L S

61  AATGCTATCTTCCGACGTGGTTATGCGGCCACGGCGGCGCAAGGGAGCGTTTCGAGCGGT
21  N A I F R R G Y A A T A A Q G S V S S G

121 GGAAGAAGTGGAGCTGTTGCTTCGGCTGTGATGAAGAAGAAGGGAGTGGAAGAATCAACC
41  G R S G A V A S A V M K K K G V E E S T

181 CAGAAGATTTCTTGGGTTCCAGATCCCAAACCGGTTATTACAGACCCGAAACCGGTTCC
61  Q K I S W V P D P K T G Y Y R P E T G S

241 AACGAGATTGACGCGGCTGAGCTACGAGCAGCTCTCTTGAACAACAAGCAGTGA
81  N E I D A A E L R A A L L N N K Q *
```

Figure 6.1. Coding nucleotide region and deduced amino acid sequence of AC3. The cDNA is 294 bp and codes for an amino acid sequence with an open reading frame of 97 amino acid residues and a molecular weight of 10.3 kDa. A protein kinase C phosphorylation site is denoted by blue amino acids. The chloroplast signal peptide is highlighted. * denotes the stop codon.

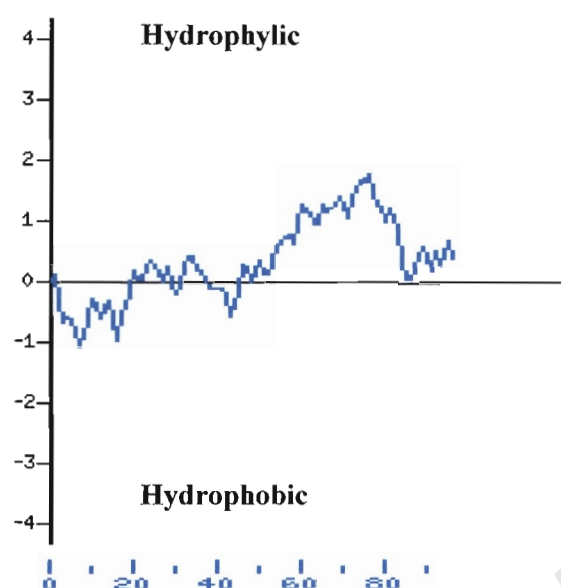


Figure 6.2. A hydropathy profile of the AC3 protein as determined by the method of Kyte and Doolittle (1982) using the Bioinformatics and Biological Computing Unit, Weizmann Institute of Science (Israel). The protein is mostly hydrophilic.

AC3	MARSISNVKIVSAFVSRELSNAIFRRGYAATAAQGSVSSGGRSGAVASAVMKKKGVEEST	
L. esc.	MARSFSNSKTLSAFVSDSVSAFLSRRGYAA-ASQGAVSGVAKG-VPRSNVMMQKSGEESV	
S. dem.	MARSFSNSKILSAFVSDSVSAFLSRRGYAA-ASQGAVSGVAKGGVPRSNVMMQKSGEESV	
N. tab.	MARSFSNSKLISAFVVDTVSSSFVRRGYAA-ASSASVPGGVRG--SGVNIMMKKWEESK	
A. mon.	MARSFTNAKVLVSVLNRFSSSLTRRGYAAAS-ATQSAKRGGV-VSISGKMAPKSGEELK	
Mung B.	MARSFTNVKVL SALVADGFSNTTTRHGFAAAAAATQSATRGG--ASIGGNMVPKSGEELK	
	**** . * * . * * * . * . * . * . * . * . * . * . * . * . * . * . * . * . * . *	
AC3	QKI----SWVPDPKTGYRPE TGSNE IDAAELRAALLNNKQ----	100%
L. esc.	KT----SWVPDPVTGYRPE GQANE IDAAELRKMLLKHRSGS-N	64%
S. dem.	KT----SWVPDPVTGYRPE GQANE IDAAELRKMLLKNRSGS-N	63%
N. tab.	KTT----SWVPDPVTGYRPE SHAKE IDAAELRQMLLNHKPRQH-	58%
A. mon.	RATTDKVS WVPDPVTGYRPE N-IKE IDVAELRATLLGKKF--N-	53%
Mung B.	RGG-EKVS WVPDPVTGYRPE N-TNE IDVADM RATVLGKKFNQ--	53%
	. ***** ***** ** * * * * . * . * . * . * . * . * . * . * . *	

Figure 6.3. Multiple sequence alignment of LEA protein group 3 sequences from tomato (*L. esculentum*; TO7161), potato (*S. demissum*; CAA66948), tobacco (*N. tabacum*; AAC06242), *A. mongolicus* (AAW31666) and mung bean (*V radiata*; T10900) with AC3 (in blue). Asterisks (*) denote identities and dots (.) denote similarities

6.3.2 Expression of AC3 in different Parts of Arabidopsis

The first LEA proteins were identified in seeds, and hence its name (Wise and Tunnacliffe, 2004). However, since then several LEA-like proteins have been found

to be expressed in vegetative tissues, especially under conditions of stress (Welin et al., 1994). To determine whether *AC3* is specific to a particular tissue, the distribution of the *LEA* mRNA in different plant tissues in *Arabidopsis* was investigated. Total RNA was isolated from five different parts of the *Arabidopsis* plant: stem, leaves, flowers, immature siliques and mature seeds. cDNA was generated from each RNA sample and PCR was performed using *AC3* specific primers. Figure 6.4 shows the result after electrophoresis on a 1.0 % EtBr-stained TAE gel.

No root RNA was available for the above experiment therefore the expression of *AC3* in roots could not be determined by RT-PCR. It appeared that *AC3* was expressed almost evenly in all vegetative tissues but comparatively low expression was found in both dry and imbibed mature seeds. An equal amount of RNA was used from each tissue for PCR and the nucleotide band obtained corresponded to the size of *AC3* (~300 bp). For more accurate quantification, northern analysis was performed using *AC3* cDNA as a probe and investigating the following tissues: leaves, stem, flowers, immature siliques, roots and mature dry seeds. While no expression of the steady-state *AC3* mRNA could be detected in stems, leaves and dry mature seeds under normal conditions, relatively high expression was present in roots (Fig. 6.5). Expression was also present in flowers and immature siliques, but not as high as in the roots. The RNA used in both the RT-PCR and the northern analysis were from the same tissue samples. However, the results do not correlate exactly. The higher sensitivity of the RT-PCR technique as compared to northern analysis might explain the difference in the results.

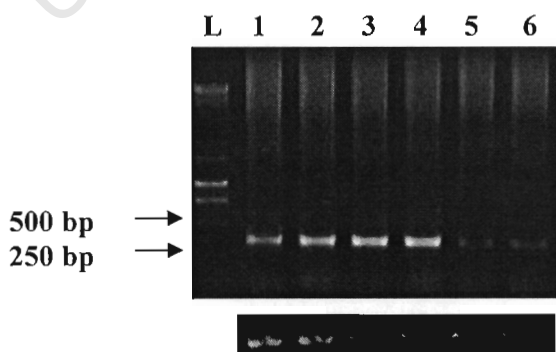


Figure 6.4. Visualization on 1.0% TAE EtBr-stained agarose gel of the expression of *AC3* mRNA in 5 different parts of the *Arabidopsis* plant by RT-PCR. Lanes 1-6: 1 kb ladder (Fermentas, UK); stem; leaves; flowers; immature siliques; dry mature seeds; imbibed mature seeds. Lower panel represents *actin* control.

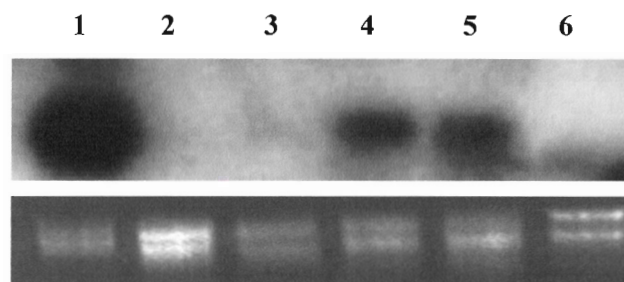


Figure 6.5. Northern blot analysis of *AC3* expression in different tissues of Arabidopsis. 1: roots; 2: stem; 3: leaves; 4: flowers; 5: immature siliques; 6: dry seeds. The lower panel represents ribosomal RNA bands on an EtBr-stained 1.2% agarose gel for comparison of RNA loading in gels.

6.3.3 Photosynthetic Efficiency of WT Arabidopsis subjected to Dehydration

Prior to the investigation of the expression of *AC3* in Arabidopsis subjected to dehydration stress, the ability of the plant to photosynthesize while under such a stress was investigated. This experiment was performed so that the kinetics between *AC3* induction during dehydration and photosynthetic efficiency during dehydration could be uncovered. Plant uses photosynthesis to convert the energy of light into the chemical energy of organic molecules. This process consists of many chemical and physical reactions involving biochemical components which enable exploitation of solar energy. The rate of photosynthesis of a plant is a good measure of water stress. As plant cells progressively lose water, photosynthetic CO_2 assimilation (A), as well as stomatal conductance (g_s) decrease and A is eventually inhibited (Lawlor and Cornic, 2002). Limitation of CO_2 supply due to reduced g_s is responsible for the lower rate of A . The metabolism of the plant is progressively impaired, caused by decreased ribulose biphosphate (RuBP) synthesis, which in turn is impaired by ATP synthesis (Lawlor DW, 2002). Limited ATP synthesis, as well as a large reductant state, alters ionic balance, protein synthesis promotes the accumulation of ‘stress’ metabolites. Therefore photosynthetic efficiency is a good physiological measure of stress in plants. Using a multi-chamber Infra-Red Gas Analyser (IRGA), several replicates could be measured simultaneously. The dehydration experiment was carried out on 5-week old WT Arabidopsis (Col 0) plants. The soil was not watered over a period of 10-15 days, until the plants looked completely dehydrated. At the beginning and end of the dehydration treatment as well as intermittently, the photosynthetic efficiencies of the plants were measured and SWC monitored. Photosynthetic efficiency was

determined by measuring gas exchange of the attached leaves of plants firstly over a range of CO₂ concentration and secondly over a range of light intensities. The former monitors carboxylation (driven by rubisco) while the latter measures quantum efficiency. Under fully hydrated conditions (62.1% SWC), WT Arabidopsis (Col 0) had an assimilation rate (A) of $\pm 15 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Fig. 6.6).

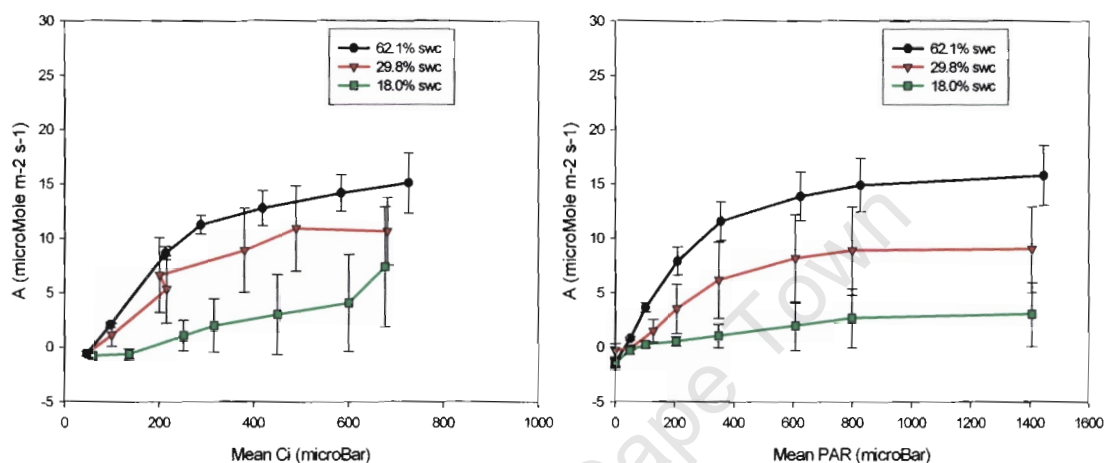


Figure 6.6. Mean gas exchange activities of dehydrating WT Arabidopsis (Col 0). Each data point is the mean of six individual plants.

As the plants started dehydrating, both carboxylation and quantum efficiency were affected. Although Arabidopsis does not lose its chlorophyll while dehydrating, at very low SWC gas exchange was at its minimum and the photosynthetic apparatus became impaired and shut down. After the last photosynthetic measurements, the plants were watered and within 24 hours they were fully rehydrated and were photosynthesising (Data not shown). Therefore the plants were not dead at 18% SWC.

6.3.4 Expression of *AC3* in Dehydrating WT Arabidopsis

AC3 was isolated because its expression conferred H₂O₂ tolerance to Baker's yeast. It has been reported that the expression of a stress-inducible gene is often not confined to a single type of stress (Ingram and Bartels, 1996). Therefore the expression of *AC3* under dehydration stress was investigated. *AC3* steady-state mRNA expression in fully hydrated leaf tissue was minimal compared to its expression when the leaves are completely dehydrated (12% and 18% SWC; Fig. 6.7). Although the loading of total

RNA on the gels were not equal in all lanes, it was evident that *AC3* steady-state mRNA was highly induced under dehydration stress. In experiment 1, at 12% SWC (Experiment 1- lane 6), very high expression was observed as compared to fully hydrated (Experiment 1- lane 1). This observation was confirmed in experiment 2, where relatively high expression of *AC3* was observed at 18% SWC (Experiment 2- lane 3) as compared to fully hydrated tissue (Experiment 2- lane 1). To investigate whether this over-expression was reversed when *Arabidopsis* rehydrated, the plants from experiment 2 were re-watered after the dehydration treatment. 72 hours after rehydration, most plants had recovered. Almost no expression of *AC3* was observed in rehydrated leaf tissues (Experiment 2- lane 4).

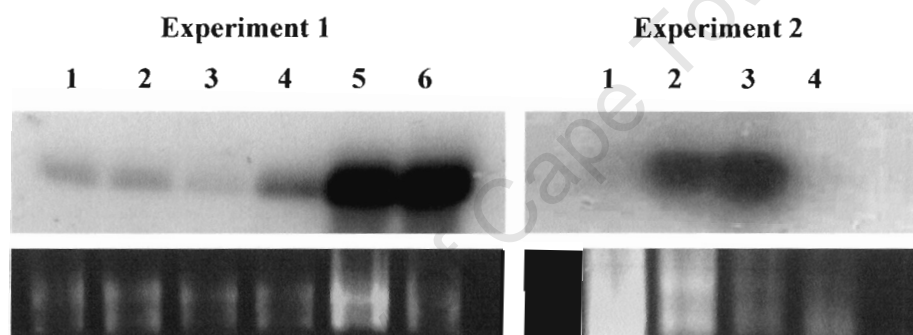


Figure 6.7. Northern blot analysis of *AC3* steady-state mRNA expression under dehydration stress in WT *Arabidopsis* (Col 0). Experiment 1- lanes 1-6: 65.0%, 53.0%, 46.0%, 33.4%, 22.8% and 12.0% SWC respectively. Experiment 2- lanes 1-4: 62.1%, 29.8%, 18.0% SWC and fully rehydrated respectively. Lower panels represent ribosomal RNA bands on an EtBr-stained 1.2% agarose gel for comparison of RNA loading in gels.

6.3.5 Expression of *AC3* under ABA and Oxidative stresses in WT *Arabidopsis*

The expression of *AC3* under dehydration stress has been established and it is a dehydration stress-inducible gene. Shinozaki and Yamaguchi-Shinozaki (1997) have previously established that the signal transduction pathway leading to dehydration stress as well as various other abiotic stresses, can either be ABA-dependent or ABA-independent. To shed some light onto which of these two pathways *AC3* expression belongs, its expression was investigated in plants treated exogenously with the ABA stress hormone. Five-week old WT *Arabidopsis* (Col 0) plants were sprayed with 100 μ M ABA. Leaves were sampled 0, 2, 4, 6, 12 and 24 hours after the start of the

treatment. The RNA isolated from the leaves was used in a northern blot analysis. Induction of *AC3* can be detected 4 hours after the start of the treatment (Fig. 6.8). This level of expression stayed relatively constant until 24 hours after start of treatment, when the level of *AC3* expression increased. This, therefore, suggests that the *AC3* gene expression is part of the ABA-dependent stress-signalling pathway. Alternatively, given the lateness of the response, the accumulation observed might reflect a physiological alteration brought about by ABA, which requires *AC3*, i.e., a secondary effect.

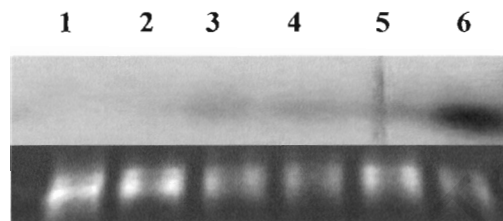


Figure 6.8. Northern blot analysis of *AC3* steady-state mRNA expression under ABA stress in 5-week old WT Arabidopsis (Col 0) plants. Lanes 1-6: 0, 2, 4, 6, 12 and 26 hours after start of the treatment respectively. Lower panels represent ribosomal RNA bands on an EtBr-stained 1.2% agarose gel for comparison of RNA loading in gels.

Because *AC3* was isolated for its ability to confer H_2O_2 to yeast, it is probable that *AC3* either has antioxidant capacities or is involved in the pathway leading to detoxification of ROS generated by oxidative stress. This may also imply that *AC3* is involved in other processes such as the protection of proteins during stress, repair, or even signalling. To investigate whether steady-state *AC3* mRNA transcript was induced by oxidative stress, 4-week old WT Arabidopsis (Col 0) plants were subjected to oxidative stress by the exogenous applications of five different pro-oxidants, namely diamide, H_2O_2 , menadione, paraquat and t-BOOH. The toxic solutions, as well as a water control, were individually sprayed on the surface of the leaves of the plants. Before the start of the treatment ($T=0$), low expression of the *AC3* steady-state mRNA transcript was observed (Fig. 6.9, lane 1). At 12 hours after the start of treatment, induction of the *AC3* transcript was observed for all the pro-oxidants except t-BOOH. However, induction was also observed in the water control at 12 hours. This could be due to a flooding response in the plants as all the leaves were well coated with the solutions after spraying. Alternatively, this could be due to

an internal rhythm of *AC3* expression within the plant cells (such as a diurnal rhythm). This aspect will be investigated in the future. In the present case, since all the plants were of the same age and were treated and harvested at the same time, we consider the result to be comparable. Since the level of induction of *AC3* steady-state mRNA in plants sprayed with diamide (thiol cross-linking agent acting on non-glutathione thiols to induce apoptosis; Zamzami et al., 1998) and plants sprayed with H_2O_2 (mainly induces programmed cell-death; Gechev and Hille, 2005), were similar to the one observed in water control plants, it is safe to assume that these two pro-oxidants do not significantly induce *AC3*. t-BOOH did not affect *AC3* expression at all. Menadione on the other hand, slightly induced *AC3* expression. Paraquat highly induced *AC3* steady-state mRNA expression. This indicates a possibility that *AC3* is induced during cell death or senescence, as well as during oxidative stress.

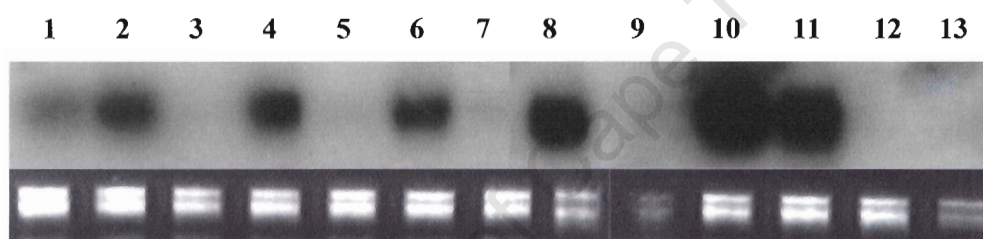


Figure 6.9. Northern blot analysis of *AC3* steady-state mRNA expression under oxidative stress in 4-weeks old WT Arabidopsis (Col 0) plants. Lanes 1-13: T=0, T=12 h water control, T=24 h water control, T=12 h diamide (1 mM), T=24 h diamide, T=12 h H_2O_2 (10 mM), T=24 h H_2O_2 , T=12 h menadione (50 μ M), T=24 h menadione, T=12 h paraquat (100 μ M), T=24 h paraquat, T=12 h t-BOOH (0.5 mM) and T=24 h t-BOOH respectively. Lower panels represent ribosomal RNA bands on an EtBr-stained 1.2% agarose gel for comparison of RNA loading in gels.

6.3.6 Investigation of the Signal Transduction Pathway of *AC3* Expression

ABA is a plant growth regulator hormone involved in many physiological and developmental processes such as transpiration, germination, dormancy and adaptation to environmental stresses (Rock and Zeevaart, 1991). ABA is induced by many environmental stresses and is responsible for the induction of many stress-responsive genes (Chaves et al., 2003). ABA mutants of Arabidopsis have been developed which are deficient in ABA biosynthesis and sensing (Leon et al., 1996). In this signalling study, two Arabidopsis ABA mutants were used: the *aba1-1* and the *abi1-1* mutants.

The *abal-1* mutant contains a mutation in the zeaxanthin epoxidase enzyme which catalyses the first of the two steps of the biosynthetic pathway of ABA (Audran et al., 2001). *abal-1* mutant plants have stunted phenotypes, even when well-watered. The phenotype can be rescued to a certain extent by exogenous application of ABA. Since *abal-1* mutant plants cannot synthesize ABA, it is expected that during stress, ABA production will not be induced and there will be no downstream induction of exclusively ABA-dependent genes. The second mutant used was an ABA insensitive mutant *abil-1*. Both *abil-1* and *abi2-1* mutants are (semi)-dominant mutations resulting in an equivalent Gly-Asp amino acid substitution in type 2C protein phosphatase (Allen et al., 1999). This mutation results in a 'whilty' phenotype as ABA activation of anion channels is impaired in guard cells. As a result, stomata do not close. Such mutants do not display a wilt-type response to desiccation or exogenous ABA application. If the expression of the *AC3* gene is solely via the ABA-dependent stress response pathway, then the mRNA should not be induced in either the ABA-deficient or the ABA-insensitive mutants, even under dehydration stress.

Under fully hydrated conditions, similar levels of *AC3* steady-state mRNA transcript were detected in WT-Ler Arabidopsis, *abal-1*, as well as *abil-1* mutants (Fig. 6.10). Although we have previously observed very little to almost no expression of *AC3* in fully hydrated conditions, occasionally and under no apparent stress condition, *AC3* expression was detected in Arabidopsis leaves (Fig. 6.10, hydrated WT-Ler). The northern analysis revealed that under dehydration stress, *AC3* was induced in WT-Ler, as previously observed in WT Col 0 Arabidopsis (section 6.3.4). In both types of Arabidopsis ABA mutants, *AC3* was induced by dehydration similar to the WT. Additionally, *AC3* mRNA induction between the two mutants was different. *AC3* expression in the dehydrated ABA-deficient mutant *abal-1* was more pronounced than in the dehydrated ABA-insensitive mutant *abil-1*.

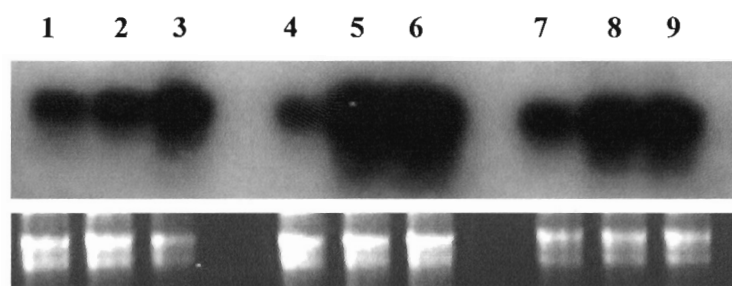


Figure 6.10. Northern blot analysis of *AC3* steady-state mRNA expression under hydrated and dehydration stress in WT Arabidopsis (Ler) and in *abal-1* and *abil-1* mutants. Lanes 1-9: WT (47.1% SWC), WT (39.1% SWC), WT (12.1% SWC), *abal-1* (48.9% SWC), *abal-1* (38.8% SWC), *abal-1* (13.5% SWC), *abil-1* (48.6% SWC), *abil-1* (38.7% SWC), *abil-1* (13.3% SWC). Lower panels represent ribosomal RNA bands on an EtBr-stained 1.2% agarose gel for comparison of RNA loading in gels.

6.4 DISCUSSION

LEA proteins in both animals and plants are associated with tolerance to water stress as a result of dehydration (Goyal et al., 2005). However, the precise role of LEAs has not yet been defined. They are assumed to protect cellular or molecular structures from the damaging effects of water loss (Cumming, 1999).

A total of 37 genes classified as putative LEAs have been identified in the Arabidopsis genome (<http://www.tigr.org/tdb/>). *AC3*, a gene from Arabidopsis, was chosen because of its ability to complement a yeast mutant susceptible to oxidative stress by detoxification of H_2O_2 . Sequence analysis of the *AC3* cDNA revealed that it codes for a LEA-like protein belonging to the group 3 LEAs out of at least six different groups of LEA proteins identified. LEA proteins are produced in abundance during seed development and can comprise up to 4% of cellular protein (Roberts et al., 1993). Using northern blot analysis, the distribution of the steady-state mRNA level of *AC3* was investigated in six different parts of the Arabidopsis plant not subjected to any stress conditions (Fig. 6.5). A relatively high abundance of the transcript was found in roots as compared to a lower expression in flowers and immature siliques. No expression was detected in either stems or leaves. Contrary to most LEA proteins, no expression was detected in mature dry seeds.

The result of an Affymatrix expression data for *Arabidopsis* (whole-genome chip; The college of Biological Sciences, University of Minnesota) is shown in Figure 6.11 illustrating *AC3* (At4g02380) compared with another group 3 LEA from *Arabidopsis* (At1g02820) which is most closely related to *AC3* at the amino acid level (62% identity). The data shows that *AC3* was present in high quantities in mature pollen, was induced by ozone (500 ppb for 6 hours) and moderately by the osmoticum glucose (30 mM for 8 hours) and by pathogen attack (nematode *Heterodera schachtii*). Higher levels of the transcript were present in the lateral roots than in control seedlings (whole RNA). *AC3* was not induced by cold (4°C for 24 hours). Although there is ~62% identity at the amino acid level between *AC3* and its closest homologue, the latter displays a very different expression profile (Fig. 6.11B). At1g02820 expression was very low throughout the plant and was not induced by any of the treatments except by low temperature stress. In this particular Affymetrix expression data, expression in seeds was not investigated.

We postulate that *AC3* plays a protective role in roots and flowers under normal conditions. In conditions of drought, roots are one of the very first organs to sense and respond to the lack of water. Similarly, the delicate inflorescence structures and reproductive organs are very susceptible to water loss and are one of the first organs affected by water stress. The expression of *AC3* in both these organs even before the onset of stress might be a precautionary measure in *Arabidopsis*.

Northern blot analysis was used to investigate the *AC3* steady-state mRNA expression in *Arabidopsis* leaves under conditions of dehydration stress and under the exogenous application of the stress phytohormone ABA as well as five different pro-oxidant compounds. Dehydration stress highly induced *AC3* expression in the leaves (Fig. 6.7). Very low to no expression was detected when the plants were fully hydrated at ~60% SWC. As dehydration proceeded, the *AC3* transcript became highly induced below ~30% SWC. The photosynthetic efficiency of WT *Arabidopsis* as it dehydrated was monitored using a gas exchange analyser.

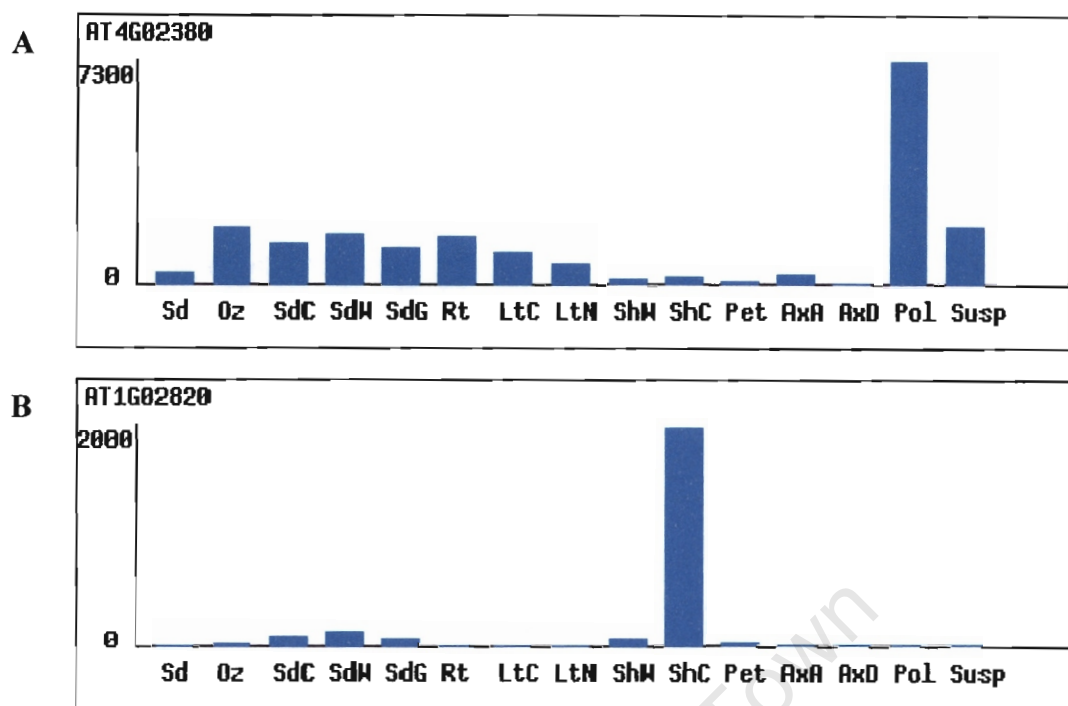


Figure 6.11. Affymetrix expression data for Arabidopsis on whole-genome chip. Sd- control 2 weeks old Col 0 seedlings, whole plant RNA; Oz- 2 weeks old Col 0 seedlings, 500 ppb ozone for 6 hours, whole plant RNA; SdC- 1 week old Col 0 seedlings, constant light, whole plant RNA; SdW- control 1 week old Col 0 seedlings, constant light, water for 8 hours, whole plant RNA; SdG- 1 week old Col 0 seedlings, constant light, 30 mM glucose for 8 hours, whole plant RNA; Rt- Primary roots from Col 0 in phytigel with 1% sucrose; LtC- Control 42 days old Col 0 lateral roots; LtN- 42 days old Col 0 lateral roots, harvested 21 days after nematode infection; ShW- Control Col 0 shoots; ShC- Col 0 shoots, 4°C for 24 hours; Pet- Col 0 petioles; Axa- Col 0 active axillary buds; AXD- Col 0 dormant axillary buds; Pol- Col 0 mature pollen; Susp- Control Col 0 suspension cells. **A:** AC3 (At4g02380). **B:** closest AC3 homologue (At1g02820).

The kinetics of *AC3* induction during dehydration corresponded with drought inhibition of photosynthesis (Fig. 6.6). It is therefore possible that upon the onset of drought, root-to-shoot signalling leads to the induction of *AC3* in leaves under dehydration stress. The *AC3* protein might be involved in the regulation of photosynthesis or in the protection of proteins involved in photosynthesis under such stresses. After 48 hours of rehydration, the transcript level had returned to a non-detectable level and normal photosynthesis of the plant had resumed (data not shown).

Exogenous application of the ABA stress hormone (100 μ M) induced expression of the *AC3* steady-state mRNA transcript (Fig. 6.8). The induction seemed to be moderate and slow over a 24 hour period (length of treatment). In a microarray experiment on full-length Arabidopsis cDNAs, *AC3* transcript was found to be induced \sim 2-fold by ABA application (50 μ M) after 24 hours (Seki et al., 2002). Therefore the induction of the *AC3* gene is ABA-dependent.

To investigate whether *AC3* is involved in oxidative stress, its expression was investigated in Arabidopsis leaves treated independently for 24 hours with diamide (1 mM), H₂O₂ (10 mM), menadione (50 μ M), methyl viologen (100 μ M) and tert-butylhydroperoxide (0.5 mM) (Fig 6.9). *AC3* steady-state mRNA expression was strongly induced by superoxide (paraquat) but only moderately by H₂O₂ and not by organic hydroperoxides. *AC3* might therefore be involved in the protection of proteins from oxidative damage induced by superoxides.

For further insight into the signal transduction pathway of *AC3*, its expression under dehydration in the Arabidopsis ABA mutants *abal-1* (ABA-deficient) and *abil-1* (ABA-insensitive) was investigated. *AC3* steady-state mRNA transcript increased considerably under dehydration stress in both mutants similar to the WT (Fig. 6.10). Expression was higher in *abal-1* than in both wild type and *abil-1*. Although an ABA stress treatment showed that *AC3* can be induced by ABA, its expression is not exclusively ABA dependent. We postulate that under conditions of dehydration stress, another factor (x) is responsible for the induction of *AC3* in an ABA-independent manner. In WT Arabidopsis plants, ABA is also induced under dehydration stress and hence both ABA and factor x contribute to *AC3* induction under dehydration. Due to the relatively low induction of *AC3* by ABA stress as compared to dehydration stress, we also postulate that factor x is the stronger and primary inducer of *AC3* via an ABA-independent pathway.

To investigate the expression of *AC3* under other stresses, a search of an Arabidopsis whole genome Affimatrix expression available online was carried out (<http://cbs.umn.edu/cgi-bin/jward/sampl2.cgi>). Figure 6.12 illustrates the Affymetrix data of *AC3* expression in shoot and roots of Arabidopsis under control conditions and

under four stresses: osmotic, UV-B, heat and salt. The high level of expression of *AC3* in roots as compared to shoots under control conditions was clear. The parameters of the stresses imposed can be found on the following website: <http://www.arabidopsis.org/info/expression/ATGenExpress.jsp>, The result gives an indication of the expression pattern of *AC3*. As confirmed by our own results, osmotic stress highly induced *AC3* in the shoots. UV-B exposure also induced *AC3*, although the expression was not maintained for 24 hours but started to decline after 3 hours and returned to normal levels after 24 hours. Both osmotic and UV-B stresses lead to the production of superoxides where *AC3* might be involved in protecting proteins from attack. Neither heat stress nor salt stress led to significant changes in the expression of *AC3* in the Affymetrix expression data. It is possible that the stresses used were not stringent enough for *AC3*. Another possibility is that *AC3* expression is highly specific to certain stresses only.

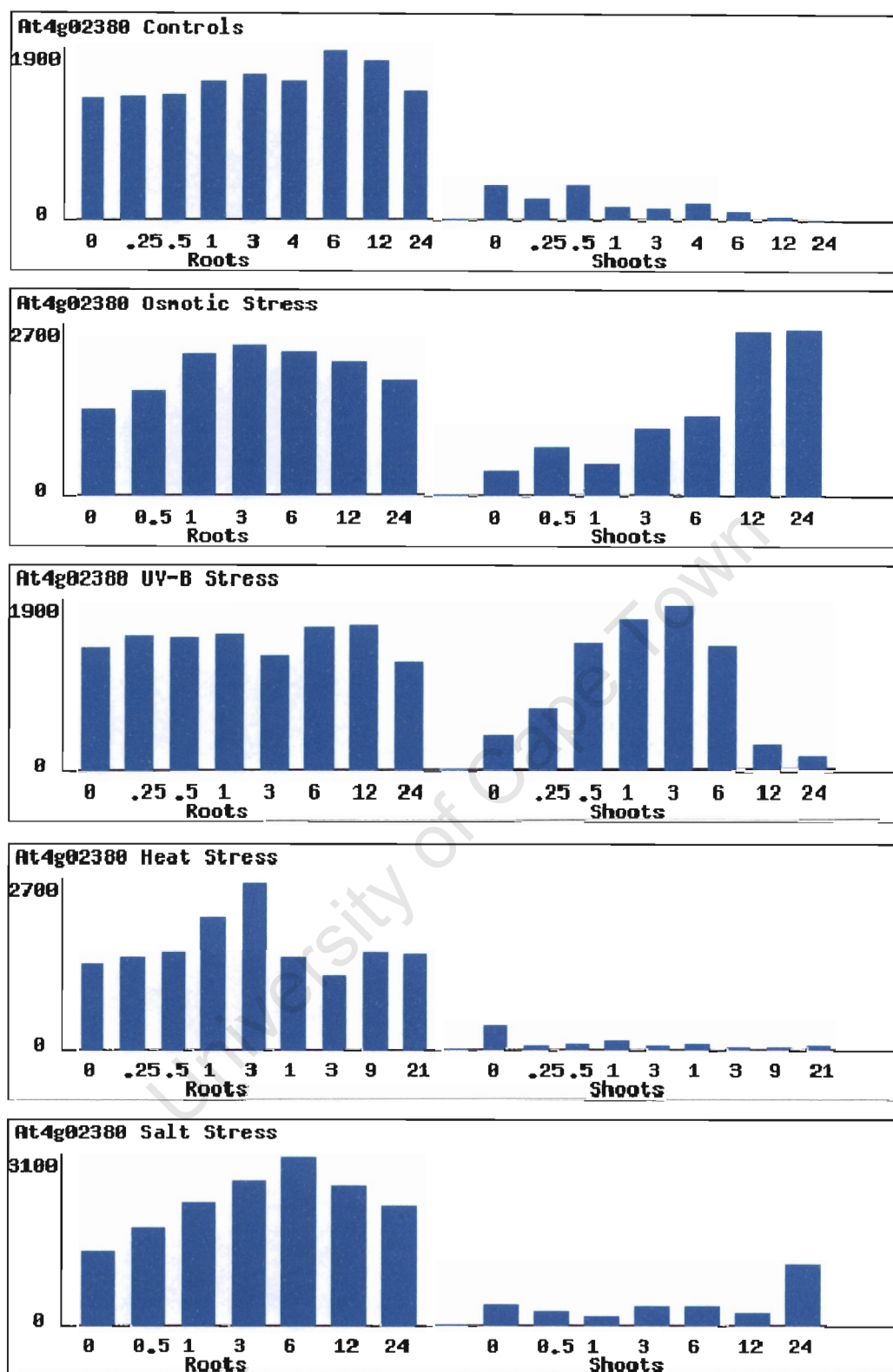


Figure 6.12. Affymetrix expression data for Arabidopsis on whole-genome chip of AC3 (At4g02380) in roots and shoots under osmotic stress, UV-B stress, heat stress and salt stress as compared to wild type. Exact parameters of stress were not specified (<http://cbs.umn.edu/cgi-bin/jward/sampl2.cgi>).

CHAPTER SEVEN

INVESTIGATING THE FUNCTION OF *AC3*

SUMMARY

The *AC3* cDNA, cloned into the yeast shuttle vector pFL61, was transformed into the oxidative stress sensitive *Δyap1* mutant yeast Y10569. Spot tests of serially diluted transformed cells on SD-U plates containing the pro-oxidants H₂O₂, diamide, menadione and ter-butylhydroperoxide showed *AC3* increased the mutant's tolerance to oxidative damage caused by diamide, menadione and ter-butylhydroperoxide. The genotype of segregating T₃ mutant plant lines with the *AC3* gene knocked out by T-DNA insertion was determined using PCR. Homozygous *AC3* knock-out (T-DNA insertion in both chromosomes) and WT (no T-DNA insertion) plants were identified. Southern blot analysis revealed no T-DNA inserts in the genome of the WT plants while multiple insertions were present in the homozygous *AC3* knock-outs. Under conditions of dehydration, *AC3* steady-state mRNA level was increased in the WT but absent in knock-out plants. Under hydrated conditions, the homozygous *AC3* knock-out plants had better photosynthetic efficiencies than WT plants. However, this was reversed as the plants dehydrated. For transgenic studies the *AC3* cDNA was cloned into the plant binary vector pBIN19-35S, in the sense orientation, to produce *AC3* over-expressor plants, and in the antisense orientation, to produce *AC3* antisense lines. Agrobacterium mediated transformation of WT Col 0 Arabidopsis plants was performed and transformants were selected on MS plates containing kanamycin. PCR and northern blot analysis were used to firstly verify the integration of the transgene into the genome, and secondly to find the level of expression of the transgene mRNA in individual plant lines.

7.1 INTRODUCTION

The investigation of the biological functions of proteins is an active area of research. In the last decade, due to major progress in biotechnology, the output of genetic information of various genomes of interest has been overwhelming. In the area of plant genome research, with the availability of the entire Arabidopsis genome sequence (www.tigr.org), one of the new challenges is to uncover the function of more than 25,000 genes in this model plant. Various strategies can be used for this

purpose. One of them is the characterization of loss-of-function mutants created by insertional mutagenesis. The availability of a mutant line in which the action of a known, specific gene has been disrupted gives the plant biologist a powerful tool in understanding the function of that gene. At the Salk Institute Genome Analysis Laboratory (SIGnAL), an Arabidopsis T-DNA knock-out library has been created with the help of Agrobacterium transformation (<http://signal.salk.edu>). If the sequence of a gene is known, it is possible to devise a PCR-based strategy to identify a plant where that specific gene has been disrupted by the insertion of the T-DNA. Once a specific knock-out mutant line has been identified, seeds are made available to the research community.

Another powerful tool in functional studies is complementation. During the last two decades, an ever-increasing number of molecular biologists have taken up yeast (*Saccharomyces cerevisiae*) as their primary research system. Consequently, the yeast genome has been sequenced (<http://www.yeastgenome.org/>). Although yeasts, being eukaryotes, have greater genetic complexity than bacteria, containing 3.5 times more DNA than *E. coli*, they share many of the technical advantages that usually permit rapid progress in the molecular genetics of prokaryotes. Some of the properties that make yeast particularly suitable for biological studies include rapid growth, dispersed cells, the ease of replica plating and mutant isolation, a well-defined genetic system, and most important, a highly versatile DNA transformation system. The development of DNA transformation has made yeast particularly accessible to gene cloning and genetic engineering techniques.

Structural genes corresponding to virtually any genetic trait can be identified by complementation from plasmid libraries. The yeast transcription factor Yap1, a bZIP DNA-binding protein of the AP-1 family, is an essential regulator of the H₂O₂ adaptive response which includes most cellular antioxidants and enzymes of the glutathione and pentose phosphate pathway (Kuge and Jones, 1994). Yeast cells deficient in YAP1 were found to be hypersensitive to hydroperoxides and thiol-oxidants, whereas over-expression of YAP1 conferred hyper-resistance. Therefore yeast $\Delta yap1$ mutant can be used in complementation of genes thought to play a role in conferring resistance to oxidative stress.

Another way of deciphering the function of a particular gene is to create transgenic lines of a model plant of study, such as *Arabidopsis* itself, over-expressing the gene of interest and then subjecting those over-expressor lines to various conditions, such as drought or oxidative stress, and observing and analysing their behaviour as compared to the WT plants. Additionally, particular assigned functions can be confirmed by analysing plants under-expressing the same gene, such as antisense lines. Such lines should ideally have opposite behaviour to the over-expressors.

The exact function of LEA proteins to date remains unclear. However, research on them is ongoing. The constitutive expression of a barley LEA protein, HVA1, in rice gave the second generation transgenic rice plants significantly increased tolerance to water deficit and salinity stress (Xu et al., 1996). The hypothesis that LEA proteins might function as molecular chaperones was tested by Goyal et al. (2005) using a group 3 LEA protein from a nematode. Chaperones have the ability to bind to non-native proteins maintaining them in a folded state and preventing aggregation (Ellis, 2004). Functional analyses of chaperones in heat stress assays using citrate synthase (CS), a protein susceptible to aggregation at elevated temperatures, suggest that the LEA protein does not behave as a classical molecular chaperone. This was not unexpected since no LEA protein upregulated by heat has yet been found. However, the protein was able to prevent protein aggregation and inactivation under conditions of water stress. A pea (*Pisum sativum*) group 3 LEA protein, PsLEAm, was found to be localized within the matrix space of pea seed mitochondria (Grelet et al., 2005). Application of ABA could re-induce expression of *PsLEAm* during germination. Although no expression of the gene could be detected in vegetative tissues, under severe water stress, its expression could be re-induced. The recombinant PsLEAm protein was found to protect two mitochondrial matrix enzymes during drying in an in vitro assay.

In this study, *AC3* *Arabidopsis* knock-out mutant plants were compared with WT relatives in their photosynthetic efficiencies under normal conditions and conditions of dehydration stress. Additionally, the ability of *AC3* to rescue the *Δyap1* phenotype under oxidative stress imposed by various pro-oxidants was investigated. Transgenic *Arabidopsis* Col 0 plants over-expressing *AC3* and *AC3*-antisense lines were generated.

7.2 MATERIALS AND METHODS

7.2.1 Transformation of WT and *Δyap1* Yeast with *AC3*

Using the method prescribed by Gietz and Woods (2002) for high efficiency yeast transformation, the *AC3* cDNA cloned into the pFL61 shuttle vector, pAC3, as well as vector only, were transformed into *Δyap1* mutant yeast cells (Y10569; *Mata* α ; *his3Δ1*; *leu2Δ0*; *ura3Δ0*; YML007w::kanMX4). The pFL61 vector was also transformed into WT yeast (Y10000; *Mata* α ; *his3Δ1*; *leu2Δ0*; *ura3Δ0*). A map of the pFL61 shuttle vector is represented in Figure 7.1 (Minet et al., 1992). The *AC3* cDNA was cloned between the *Bst*XI restriction enzyme sites of the vector in the antisense orientation (expression of *AC3* still occurs) with respect to the PKG (phosphoglycerate kinase) promoter.

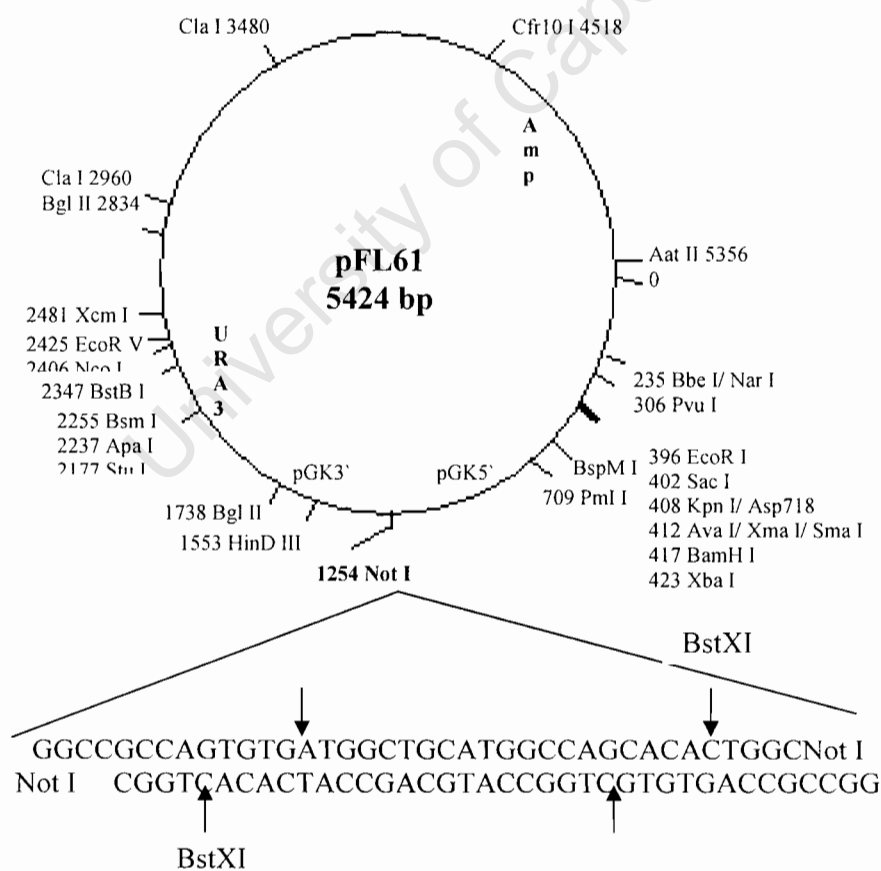


Figure 7.1. Map of yeast shuttle vector pFL61

7.2.2 Toxicity test

Δyap1 mutant yeast cells transformed with the pFL61 vector or with pAC3, as well as WT yeast transformed with pFL61, were each inoculated in 10 ml minimal media (0.17% yeast nitrogen base without amino acids, 0.5% ammonium sulphate, 2% glucose, 0.77 g/L CSM-URA) and grown at 28°C O/N with shaking. The cells were diluted 1:10-1:50 into 1 ml minimal media and read at OD₆₀₀. From this reading the number of cells/ml was estimated, with the assumption that 10⁶ cells=OD₆₀₀ of 0.1 (Gietz and Woods, 2002). The cells were diluted in sterile water to give a stock suspension of 3 × 10⁴ cells/6 μl. By making 10-fold and 3-fold dilutions, the following final concentrations were obtained: 1 × 10⁴, 3 × 10³, 1 × 10³, 3 × 10², 1 × 10² and 3 × 10 cells per 6 μl of water. Minimal media agar plates each containing varying concentrations of a pro-oxidant were prepared. The pro-oxidants used were H₂O₂, ter-butylhydroperoxide (t-BOOH), diamide and menadione. 6 μl drops of each cell dilution were pipetted onto the tox-plates which were incubated at 28°C for 3-4 days before the results were recorded. Each experiment was performed in triplicate.

7.2.3 Determining the Genotype of *AC3* Knock-out plants

Arabidopsis seeds from segregating T3 mutant plant lines with the *AC3* gene knocked out by T-DNA insertion (SALK 099663, <http://www.arabidopsis.org/servlets/TairObject?id=98011&type=polyallele>) were purchased from the Salk Institute Genome Analysis Laboratory (SIGnAL, UK) (Alonso et al., 2003). The T-DNA has been inserted in the intergenic region, in the reverse orientation to the gene, at position 190bp of the ORF. Provided that this T-DNA insert is the only one present in the genome, its position and orientation should disrupt the *AC3* gene function and produce a genuine *AC3* knock-out phenotype. The seeds were sterilized as described in section 6.2.2 and germinated on MS-media (Murashige and Skoog, 1962). After 10 days, the seedlings were transferred onto water-saturated prescription compost (prepared by Petersfield Products) which contained chloronicotinyl (Intercept; 280 g/m³) and grown in a controlled environment cabinet with conditions as follows: a temperature of 25°C, a relative humidity of 70%, a day length of 10 hours with a light intensity of 250 μmolm⁻²s⁻¹. Leaf samples from 16 surviving plants were taken and genomic DNA extracted using a protocol adapted from Edwards et al. (1991). The tissue (generally one leaf) was

ground in liquid nitrogen in a microfuge tube using a sterile plastic pestle for 20 seconds. 400 μ l of buffer (200 mM Tris-Cl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) was added and the tissue was ground further until well macerated. The tube was vortexed for 5 seconds and left at RT for 5 minutes. After centrifugation at full speed (14500xg) for 5 minutes, 300 μ l of the supernatant was transferred to a fresh tube and an equal volume of isopropanol was added. The tube content was well mixed and left at RT for 10 minutes followed by centrifugation at 14500xg for 15 minutes. The resulting pellet was rinsed in 70% ethanol and then air-dried. The DNA pellet was resuspended in 100 μ l of TE buffer (10 mM Tris-Cl pH 7.5, 1 mM EDTA). To determine the genotype of the plants with respect to the T-DNA insertion, 1 μ l of DNA isolated was used in a PCR reaction using a mixture of three different primers: LP (5'-AACTCTATCATACATCCAACGATCC-3'; left genomic primer), RP (5'-CAAAGGCCAGAATTGACGCAG-3'; right genomic primer) and LBa1 (5'-TGGTTCACGTAGTGGGCCATCG-3'; left border primer of pROK2). Figure 7.2 is a schematic illustration of the PCR reaction and possible products. WT (no T-DNA insertion) should give a product size of ~900 bp (from LP to RP). Homozygous lines (insertion in both chromosomes) should give a band of 410 + N bp (from RP to insertion site 300 – N bases, plus 110 bases from LB to the left border of the vector). Heterozygous lines (one of the pair of chromosomes with insertion) will give both bands.

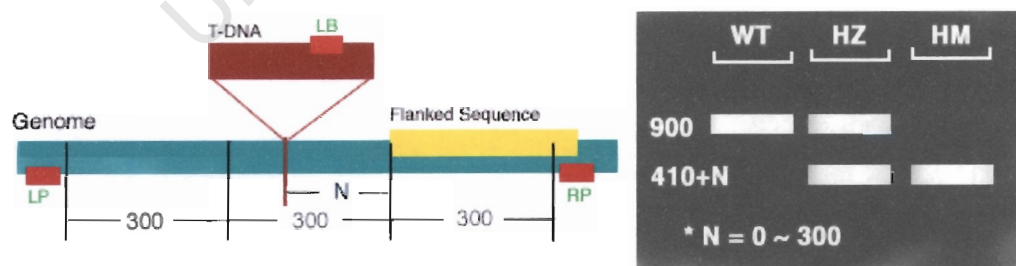


Figure 7.2. Schematic illustration of the PCR and product sizes expected when using LP, LB and RP primers for WT (wild-type; no insertion), HZ (heterozygous; one of the pair of chromosomes with insertion) and HM (insertion in both chromosomes) of a SALK T-DNA insertion line (<http://signal.salk.edu/tdnaprimers.html>)

7.2.4 Southern Blot Analysis of WT and *AC3* Knock-out plants

To determine the number of T-DNA insertions in the SALK insertion lines, Southern blot analysis was performed on both homozygous *AC3* knock-out lines as well as WT lines. Although WT lines might be free from insertion of a T-DNA in the *AC3* gene, T-DNA inserts might be integrated elsewhere in the genome, possibly disrupting a house-keeping gene, in which case such a line will have to be rejected from further analysis. Genomic DNA was isolated from four WT and four homozygous *AC3* knock-out lines using a method based on the detergent hexadecyltrimethylammoniumbromide (CTAB). Approximately 1 g of leaf tissue was ground in liquid nitrogen using a pestle and mortar. Upon thawing 1 ml of CTAB extraction buffer (2% CTAB, 0.02 M EDTA, 0.1 M Tris-Cl pH 8.0, 1.4 M NaCl, 25 mM DTT) was added. Mortared samples were poured into 2 ml microfuge tubes containing 100 µl of chloroform: octanol [24:1 (v/v)] mix, and incubated at 65°C for 30 minutes followed by 15 minutes at RT. After the addition of 1 ml chloroform: octanol mixture, samples were shaken gently until a homogenous suspension was obtained. Centrifugation for 7 minutes at 13000 rpm caused separation into two distinct layers. The upper phase (containing the DNA) was transferred to a new microfuge tube using pipette tips cut to produce a wider aperture, and 1 ml of 95% ethanol (-20°C) was added. Samples were placed at -20°C for 1 hour and then allowed to stand for a further 5 minutes at RT after incubation. Tubes were inverted to mix the buffer and ethanol and collect the DNA together. DNA strands were gathered by looping onto a hooked Pasteur pipette and immersed into an Eppendorf tube containing 1 ml of 75% ethanol/0.2M sodium acetate. The collected DNA was allowed to stand in ethanol for 10 minutes to wash. After air-drying for 5 minutes, the DNA was resuspended by immersing the hooks in 50-200 µl of TE buffer.

15 µg aliquots of each genomic DNA sample were digested with 50 units of EcoRI restriction enzyme, which does not cut either the genomic sequence of *AC3* or the T-DNA insert. Digestions were incubated overnight at 37°C and were ethanol-precipitated and resuspended in 30 µl TE buffer. The DNA samples were electrophoresed in 0.8% agarose/TAE gels at 23 V for 18-24 hours. After electrophoresis, the agarose gel was immersed in depurination buffer (0.25 M HCl) for 10 minutes, followed by incubation in denaturing solution (1.5 M NaCl, 0.5 M

NaOH) for 30 minutes and in neutralisation solution (1.5 M NaCl, 0.5 M Tris-Cl pH 7.2) for another 30 minutes, both with gentle agitation. Nucleic acids were transferred from the gel onto a nylon filter (Hybond NX, Amersham) by capillary transfer O/N using 10xSSC (1.5 M NaCl, 0.15 M tri-sodium citrate). The membrane was washed briefly with distilled water and the DNA was cross-linked with UV irradiation (Startagene StratalinkerTM).

The probe used for the Southern analysis was the T-DNA insert. The probe was labelled with ³²P-dCTP using a random primed labelling kit according to the manufacturer's instructions (Invitrogen, UK). Pre-hybridization of the Southern membrane was carried out at 65°C in pre-hybridization buffer (0.5 M NaH₂PO₄, 1 mM EDTA, 7% SDS, 1% BSA) for a minimum of 2 hours. After pre-hybridization, the radiolabelled probe was denatured (placed in a boiling water bath for 10 minutes and then cooled on ice) and then was added to the membrane. Hybridization was carried out at 60°C for 16 hours with gentle shaking. The membrane was washed twice at 60°C for 10 minutes in wash buffer B (0.1% SDS, 0.5xSSC) and autoradiographed at -70°C onto high-performance-autoradiography film (Amersham Pharmacia Biotech, USA). After 10 days exposure, the film was developed manually using developer and fixer reagents (Sigma, UK) according to manufacturer's instructions.

7.2.5 Expression of *AC3* in Dehydrated WT and *AC3* Knock-out plants

Northern analysis was used to verify whether the expression of *AC3* was indeed prevented in the knock-out plants due to disruption of its genomic sequence. Total RNA was isolated from two WT and two homozygous *AC3* knock-out replicates which had been subjected to dehydration stress. Northern blot analysis was performed as described in section 6.2.6.

7.2.6 Comparison of Steady State CO₂ Exchange in WT and *AC3* Knock-out plants

CO₂ assimilation was measured from WT and homozygous *AC3* knock-out plants under hydrated and dehydrated conditions using four replicates for each plant line. The procedure is outlined in section 6.2.4.

7.2.7 Chlorophyll and Protein Determination of WT and AC3 Knock-out plants

A leaf disc was cut out of a leaf from the middle rosette, weighed and immediately frozen in liquid nitrogen. The leaf disc was ground to a fine powder and extracted in 1 ml of 1 M perchloric acid (HClO₄; stored at 4°C). The sample was ground continuously until completely thawed. The homogenate was clarified by centrifugation for 10 minutes at 17000xg. The pellet from this was used for determining the phaeophytin concentration which can be used to determine the chlorophyll concentration (Vernon et al., 1960). The pellet was re-extracted in 10 ml of 80% acetone (v/v) and left for 16 hours at 4°C. The 10 ml extract was then mixed vigorously and 1 ml was used for centrifugation at 17000xg for 10 minutes. The absorbance of the supernatant was recorded at 655 nm (A₆₅₅) and 666 nm (A₆₆₆) and the phaeophytin concentration was calculated using the equation below:

$$\text{Phaeophytin } (\mu\text{g/ml}) = (6.75 \times A_{666}) + (26.03 \times A_{655})$$

The pellet from the last centrifugation step was resuspended in 100 µl of 0.1 N NaOH and made up to 100 µl using sterile distilled water. 50 µl of this sample was then used for determining protein content using the Bradford method (Sigma, UK). Protein content was calculated using a BSA standard curve (Bradford, 1976).

7.2.8 Cloning of AC3 in pBin19-35S in the Sense and Anti-sense orientations

Cloning of AC3-sense

Using AC3 specific primers with incorporated restriction enzyme sites (BamHI in the forward primer and XbaI in the reverse primer; primer sequences outlined in section 6.2.7), the AC3 fragment was amplified by PCR using pAC3 as template and *pfu* DNA polymerase (Promega, UK) for high fidelity amplification. The PCR reaction mixture was according to the manufacturer's instructions and the conditions were as follows: initial 94°C denaturation for 2 minutes followed by 30 cycles of 94°C for 1 minute, 53°C for 45 seconds and 72°C for 2 minutes. A final extension at 72°C was held for 5 minutes. The PCR product was loaded onto 1% agarose/TAE gel to check for size and purity. The band was excised and gel purified using the QIAquick gel extraction kit (Qiagen, UK) following manufacturer's instructions. To aid restriction

analysis of the PCR amplified fragment, the latter was subcloned into the pGEM[®]-T Easy vector (Promega, UK) following manufacturer's instructions. The pGEM[®]-T Easy vector containing the *AC3* cDNA, pGEM[®]-T Easy-*AC3* was transformed into *E. coli* DH5 α [™] competent cells (Invitrogen, UK) for plasmid amplification using the QIAprep Spin Miniprep Kit (Qiagen, UK) following the manufacturer's instructions. 5 μ g of purified plasmid was used in a double digest using 5 units of BamHI and XbaI restriction enzymes to release the *AC3* fragment. The entire restriction digest was separated on a 1% agarose/TAE gel and the \pm 300-bp *AC3* cDNA fragment was gel purified as above. Because the two enzyme sites chosen were in too close proximity on the DNA backbone of pBin19-35S (Fig. 7.3), a double digest could not be performed. Instead, 10 μ g of the pBin19-35S binary vector was digested first with 10 units of XbaI restriction enzyme, followed by ethanol precipitation of the linearized DNA, and then a second digest with 10 units of BamHI restriction enzyme, followed by another ethanol precipitation. The ligation reaction of *AC3* cDNA in the pBIN19-35S was calculated using the following formula:

$$\frac{\text{Mass of vector (ng)} \times \text{size of insert (bp)}}{\text{size of vector (bp)}} \times \text{molar ratio} = \text{mass of insert (ng)}$$

The ligation reaction was set up using T4 DNA ligase (Promega, UK) and incubated at 15°C O/N. Control ligation reactions were also set up with control 1, having no insert (checking for relegation of vector), and control 2, having no insert and no ligase (checking for unrestricted vector). All three ligation reactions were used for transformation in DH5 α [™] *E. coli* competent cells (Invitrogen, UK). Selection was on media containing kanamycin (50 μ g/ml). Colonies were initially checked by colony PCR where a small amount of cells from the colony as template. Samples were sent for sequencing (Oxford University UK) using the Dye Terminator method. The forward primer used was a 35S-promoter specific primer (5'-AGTGGTCCCAAAGATGGACC-3') and the reverse primer was an *AC3* specific primer (section 6.2.7).

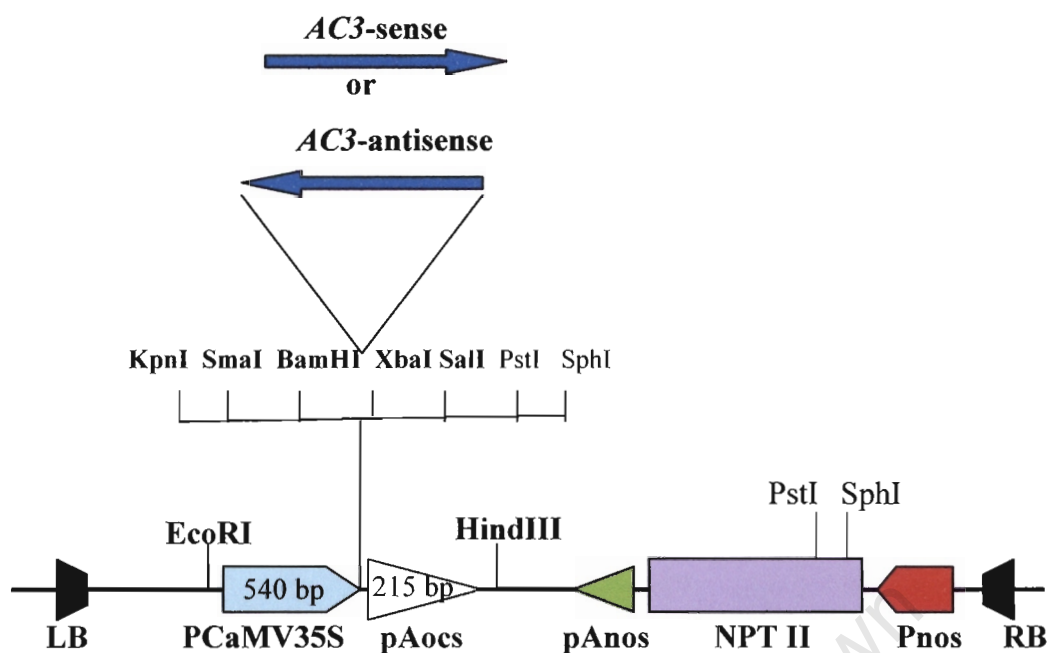


Figure 7.3. Important features of the pBIN19-35S binary vector. Restriction enzyme sites in bold letters are unique sites in the plasmid which codes for kanamycin resistance for selection in *E. coli*. The site for the *AC3* cDNA sense and antisense insertion is shown. The basis vector is pBIN19 (U09365).

Cloning of *AC3*-antisense

Primers specific to *AC3* were designed with restriction sites which would allow cloning of the *AC3* cDNA in the reverse orientation in the pBIN19-35S vector. An *AC3* forward primer was designed with an *Xba*I restriction enzyme site: 5'-CAAGTCTAGACTTACTTCGAAAATGGC-3', and an *AC3* reverse primer was designed with a *Bam*HI restriction enzyme site: 5'-CCGGATCCTCTCCTCTTAAAGACC-3'. Using those primers, the *AC3* cDNA fragment was amplified by PCR and the cloning was done as described above.

7.2.9 Preparation of Electro-competent *Agrobacterium* cells

A plate culture of *Agrobacterium* strain GV3101 (pMP90) (kindly donated by Dr Peter Buckner, Rothamsted Research, UK) was used for the inoculation of a 5 ml LB bacterial culture with rifampicin (25 µg/ml) and gentamycin (25 µg/ml). The culture was grown at 28°C O/N and used to inoculate a 500 ml culture. When the cells reached log phase (OD₅₅₀ of 0.5-0.8), the culture was chilled by standing in an ice-water bath and swirling gently. The cells were kept at 4°C for further steps. The cells

were pelleted by centrifugation at 4000xg for 10 minutes at 4°C, in a pre-chilled rotor. The supernatant was discarded and 10 ml of ice-cold sterile distilled water was added to the pellet. Using a wide-bore pipette, the cells were gently resuspended thoroughly. The suspension volume was adjusted to 500 ml with ice-cold water and the centrifugation and washing steps were repeated 4 times, resuspending the cells in 250 ml and 50 ml of ice-cold sterile distilled water respectively. The cells were pelleted again and resuspended in 5 ml of 10% (v/v) ice-cold sterile glycerol. The cells were dispensed in 50 µl aliquots and snap frozen in liquid nitrogen and stored at -70°C until use.

7.2.10 Electroporation of *Agrobacterium* cells

A 50 µl tube of competent cells was thawed on ice. Eppendorf electroporation cuvettes (800 µl capacity; Sigma, UK) were cooled at 4°C. 10 ng of DNA in 1 µl total volume was added to the cells on ice and mixed gently. The cells were pipetted onto the side of the pre-chilled cuvette and firmly tapped until the cells slipped to the bottom and spanned the whole length of the bottom of the cuvette. The cells were electroporated (2.5 V, 200 Ω, 25 µFD) using the Gene Pulser™ (Biorad, USA). Immediately 960 µl of SOC media (per 100 ml: 2 g tryptone, 0.5 g yeast extract, 1 ml 1 M NaCl stock, 0.25 ml 1 M KCl, sterile distilled water to 100 ml) was added, the cells mixed and transferred to sterile 10 ml tubes which were incubated at 29°C with rotation for 3 hours. 200 µl of the culture was plated on selective LB media (25 µg/ml gentamycin, 25 µg/ml rifampicin and 50 µg/ml kanamycin) and incubated at 30°C for 2-3 days. Individual colonies were checked for the presence of the binary vector by colony PCR where a few cells from one colony were used as template in a PCR reaction. Glycerol stocks were made of the relevant colonies (half volume of saturated culture with half volume of 50% glycerol, flash frozen in liquid nitrogen and stored at -80°C).

7.2.11 *Arabidopsis* Transformation

This method was adapted from Clough and Bent (1998) and allows the achievement of a transformation rate of above 1%. Healthy WT *Arabidopsis* (Col 0) plants were grown in a controlled environment cabinet that maintained a temperature of 25°C, a relative humidity of 70%, a day length of 10 hours with a light intensity of 250

$\mu\text{molm}^{-2}\text{s}^{-1}$ until flowering. The first bolts were clipped to encourage proliferation of secondary bolts. Plants were ready for transformation roughly 6-10 days after clipping. To prepare the *Agrobacterium* strain carrying the gene of interest, a 500 ml liquid culture was grown O/N at 28°C in LB media containing antibiotics. The OD₆₀₀ of the cells was measured before the cells were pelleted. The cells were resuspended in half-strength MS liquid media to a final OD₆₀₀ of 0.8-1.0. Approximately 500 ml of cell suspension was needed for each transformation event, and 5-10 plants were used in one transformation event. Before dipping, Silwet L-77 (LEHLE SEEDS, USA) was added to a final concentration of 0.035% and 15 $\mu\text{g/L}$ of benzylaminopurine (BAP). After mixing the cell suspension well, the above-ground parts of the plants were dipped for 5 seconds with gentle agitation. Dipped plants were placed under a cover for 24 hours to maintain high humidity. The same dipping procedure was repeated on the same plants 7 days after the first dipping to increase transformation efficiency. Plants were watered as normal until seeds became mature. The seeds were harvested and transformants were selected by sterilizing and plating on full-strength MS media with kanamycin selection (50 $\mu\text{g/ml}$). After sterilization (section 6.2.2), 40 mg (\pm 2000) seeds were resuspended in 4 ml of 0.1% agarose which were spread on MS plates. After 48 hours at 4°C, plates were transferred into a growth chamber maintained at 22°C, a relative humidity of 50%, and a day length of 16 hours and light intensity of 250 $\mu\text{molm}^{-2}\text{s}^{-1}$. After 10 days, putative transformants were transferred to soil.

7.2.12 Testing for Positive Transformants

Using PCR

Putative transformants were tested by doing PCR on genomic DNA. The method used for genomic DNA isolation is outlined in section 7.2.3. For testing *AC3*-sense transformants, primers used were the 35S-promoter primer (sequence in section 7.2.7) and the *AC3* reverse primer used for sense cloning (sequence in section 7.2.7). For testing *AC3*-antisense transformants, primers used were the 35S-promoter primer (section 7.2.7) and the *AC3* reverse primer used for antisense cloning (sequence in section 7.2.7).

Using northern blot analysis

Following testing by PCR, RNA was isolated from putative transformants and northern blot analysis was performed as outlined in sections 6.2.5 and 6.2.6. For *AC3*-antisense putative transformants, plants were subjected to dehydration stress first before RNA isolation and northern analysis.

7.3 RESULTS

7.3.1 Yeast Toxicity test- AC3 Protects against Oxidative Stress

WT yeast (Y10000) transformed with the yeast shuttle vector pFL61, and the yeast *Δyap1* (Y10569) mutant, which is hypersensitive to hydroperoxides because of the deficiency in the YAP1 transcription factor, transformed with both the vector pFL61 and the recombinant vector pAC3 containing the *AC3* cDNA, were subjected to oxidative stress by plating on minimal media containing either one of the following four pro-oxidants: H₂O₂ (0.2 mM), diamide (1.1 mM), menadione (7.5 μM) and t-BOOH (55 μM) (Fig. 7.4). Primary transformed colonies were grown in liquid medium, serially diluted, and spotted onto SD-U plates supplemented with the pro-oxidants. The *Δyap1* mutant expressing the AC3 protein was more resistant to 1.1 mM diamide, 7.5 μM menadione and 55 μM tBOOH than the *Δyap1* mutant transformed with the vector pFL61, whose growth was partially suppressed by the oxidative stress imposed. The phenotype rescue was more pronounced for menadione than for the two other pro-oxidants. Although *AC3* was isolated by its ability to confer tolerance to H₂O₂ stress in the *Δyap1* mutant, this could not be demonstrated here, despite several attempts.

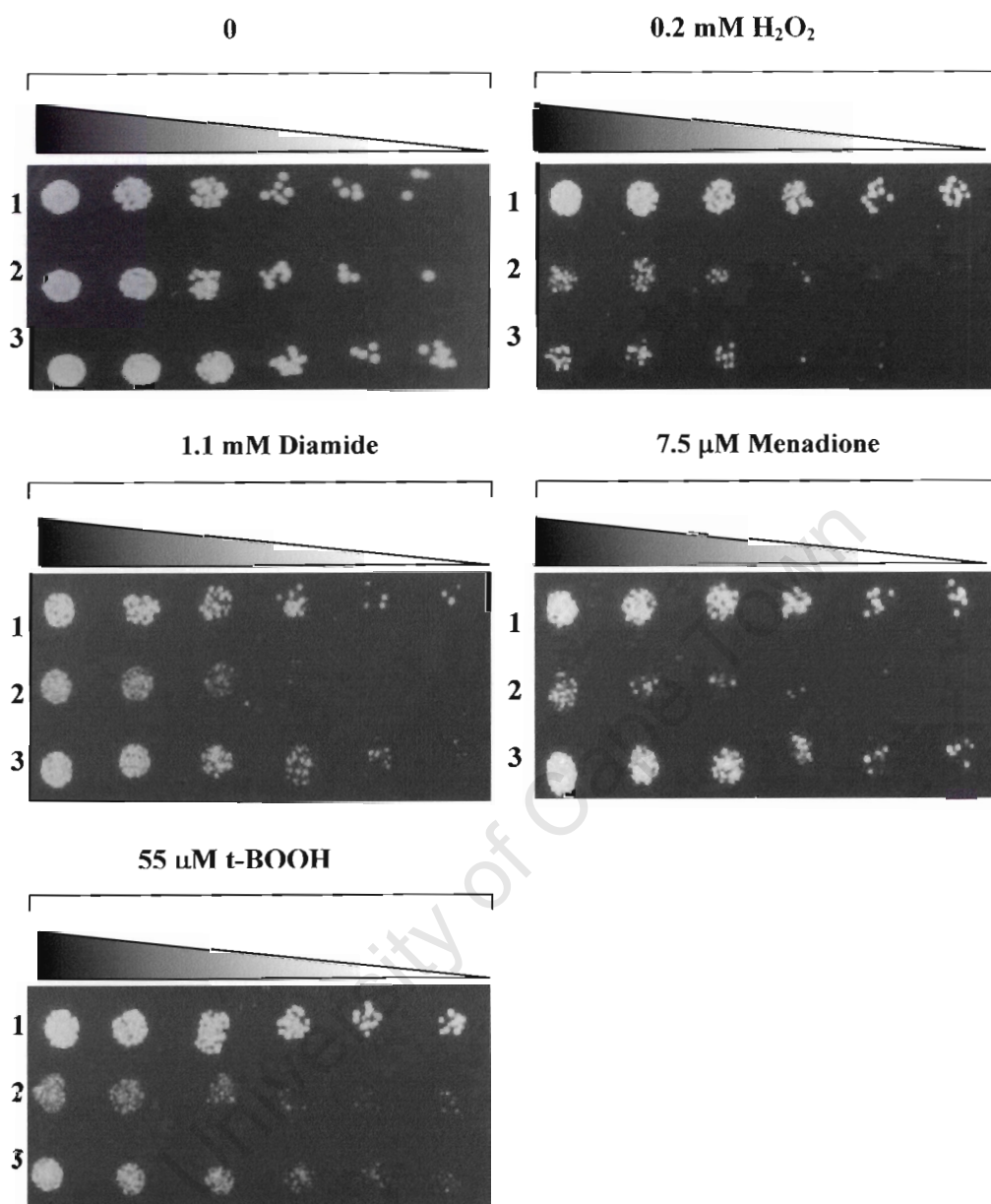


Figure 7.4. Resistance profiles of yeast producing the Arabidopsis protein AC3. The tolerance to H_2O_2 , diamide, menadione and ter-butylhydroperoxide of the *yap1*-deficient yeast strain (Y10569) was analysed transformed with pFL61 vector (rows 2) or with recombinant vector pAC3 (rows 3), compared with the WT yeast (Y1000) transformed with pFL61 (rows 1). Transformants were grown and culture serially diluted (1×10^4 , 3×10^3 , 1×10^3 , 3×10^2 , 1×10^2 and 3×10 cells per $6 \mu\text{l}$). $6 \mu\text{l}$ aliquots were spotted onto minimal media (0) or minimal media supplemented with $0.2 \text{ mM H}_2\text{O}_2$, 1.1 mM diamide, $7.5 \mu\text{M}$ menadione and $55 \mu\text{M}$ ter-butylhydroperoxide. Triangles indicate gradation from higher to lower cell densities. Plates were photographed 4 days after incubation at 30°C .

7.3.2 Genotyping of *AC3* Knock-out plants

Arabidopsis mutant seeds with the *AC3* gene knocked out by T-DNA insertion using *Agrobacterium* transformation were purchased from the Salk Institute Genome Analysis Laboratory (SIGnAL, UK). Before any analysis could be done on those plants it was important to select for those plants which were homozygous for the T-DNA insertion, i.e., which had both *AC3* genes knocked out. A PCR strategy was used on genomic DNA using a trio of specially designed primers. The results are illustrated in Figure 7.5.

Using this strategy, 16 surviving plants from the T3 generation of the *AC3* knock-out were assessed. Six plants were found to be WT, three plants were heterozygous and seven plants were homozygous. For further analysis, the seeds from the WT and homozygous plants were collected.

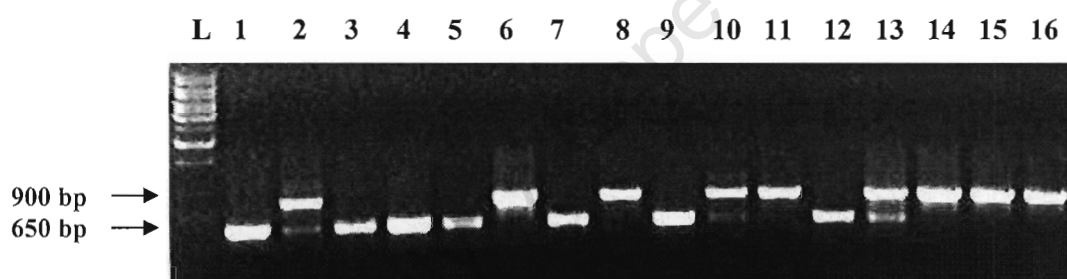


Figure 7.5. Visualization on 1.0% TAE EtBr-stained agarose gel of the genotyping of individual plants from the T3 generation of *Arabidopsis AC3* knock-out by T-DNA insertion (SIGnAL). L: 1 Kb ladder. Lanes 1-16: PCR from individual progenies of the T3 generation. WT plants are represented in lanes 6, 8, 11, 14, 15 and 16 with only one band each (900 bp). Heterozygous plants are represented in lanes 2, 10 and 13 with two bands (900 bp and 650 bp). Homozygous plants are represented in lanes 1, 3, 4, 5, 7, 9 and 12 with only one band (650 bp).

7.3.3 Southern Blot Analysis of WT and homozygous *AC3* Knock-out plants

After determination of the genotypes, the next step was to determine the number of insertions of the T-DNA in the genomes of WT and homozygous plants. This was important as although no T-DNA inserts were present in the *AC3* gene of the WT plants, the latter were the progeny of plants which were originally infected with *Agrobacterium* to produce knock-outs and therefore the possibility existed that T-

DNA inserts might have inserted elsewhere in the WT genome. The same applied for the homozygous plants as the *AC3* gene might not be the only one containing a T-DNA insert, in which case the observed phenotype might not be entirely due to the absence of *AC3*. The Southern blot analysis showed no bands for the WTs (Fig. 7.6). Those plants and their progenies can therefore be used as WT controls for further experiments. However, for the homozygous *AC3* knock-out plants, multiple bands were revealed of which three bands were more distinct than the others at positions ~15 kb, ~6 kb and ~4 kb. Because the restriction enzyme (EcoRI) used for digestion of the genomic DNA did not cut the T-DNA insert, the result meant that each band observed represented a different T-DNA insertion into the genome, one of which is the insertion into the *AC3* gene locus. The positions of the other T-DNA insertions were unknown.

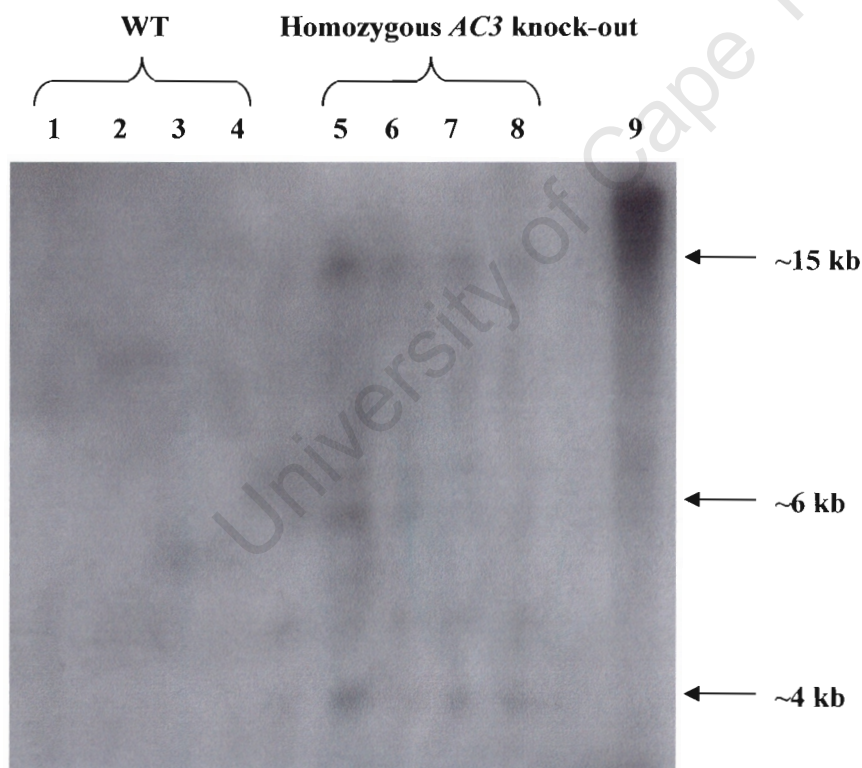


Figure 7.6. Southern blot analysis of the T-DNA insert in four WT plants and four homozygous *AC3*-knock-out plants. Fifteen micrograms (15 µg) of genomic DNA isolated from leaf tissue was digested with EcoRI, fractionated by electrophoresis, transferred on a nylon membrane and hybridized with ^{32}P -labelled T-DNA probe. Lanes 1-4: WT plants. Lanes 5-8: Homozygous *AC3*-knock-out plants. Lane 9: 10 pg of positive plasmid control. The molecular weights of the bands observed are indicated.

7.3.4 Expression of *AC3* in Dehydrated WT and *AC3* Knock-out plants

Northern blot analysis was used to investigate the expression of the *AC3* steady-state mRNA in WT and *AC3* knock-out plants subjected to dehydration stress (Fig. 7.7). Under hydrated conditions, the detection by northern analysis of the *AC3* steady-state mRNA transcript in WT Arabidopsis plants was undetected (section 6.3.4). Under dehydration stress, the *AC3* level increased dramatically. Two replicates of homozygous *AC3*-knock-out plants were dehydrated by not watering the soil and their *AC3* expression was investigated. This was compared with two replicates of dehydrated WT plants. As expected, no *AC3* steady-state mRNA expression was detected in either replicates of the homozygous *AC3* knock-out plants. In both replicates of the WT plants, *AC3* expression was induced.

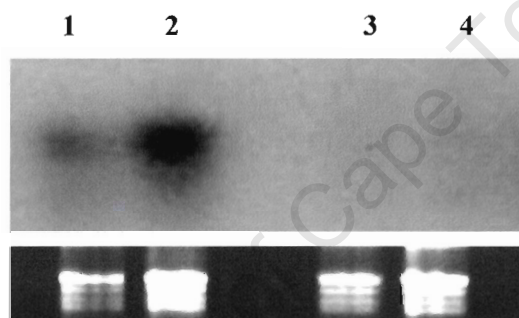


Figure 7.7. Northern blot analysis of *AC3* steady-state mRNA expression under dehydration stress in WT and homozygous *AC3* knock-out Arabidopsis plants. Lanes 1 and 2: two replicates of WT plants; lanes 3 and 4: two replicates of homozygous *AC3* knock-out plants. Lower panels represent ribosomal RNA bands on an EtBr-stained 1.2% agarose gel for comparison of RNA loading.

7.3.5 Gas Exchange Measurements in WT and *AC3* Knock-out plants

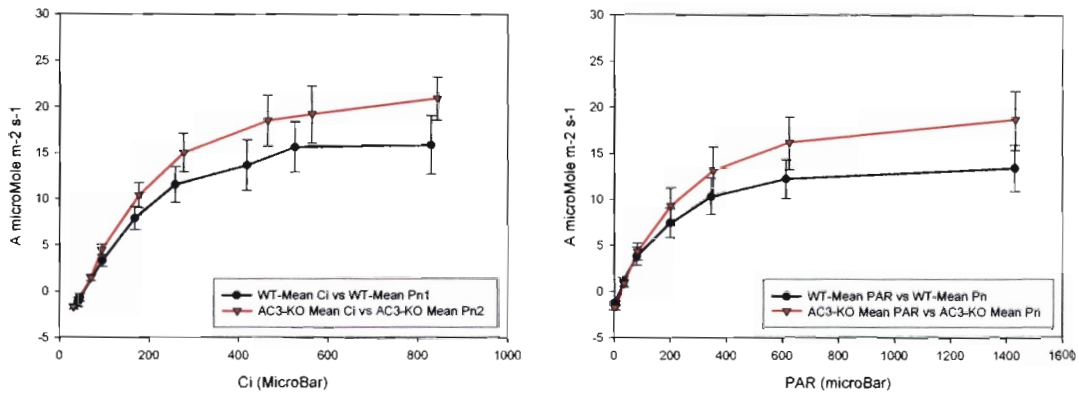
The ability of *AC3* knock-out plants to photosynthesize under normal conditions, as well as under conditions of dehydration stress, was measured. This was compared to WT plants. The experiment was done using a multi-chamber Infra-Red Gas Analyser (IRGA). All replicates (four for each plant type) were grown for five weeks and measured simultaneously. The dehydration experiment was carried out by withholding water from the soil until the plants looked completely dehydrated. At the beginning, mid-way and at the end of the dehydration treatment the photosynthetic efficiencies of the plants and SWC were measured. Photosynthetic efficiencies were

measured by measuring gas exchange of the attached leaves of plants firstly over a range of CO₂ concentration and secondly over a range of light intensities.

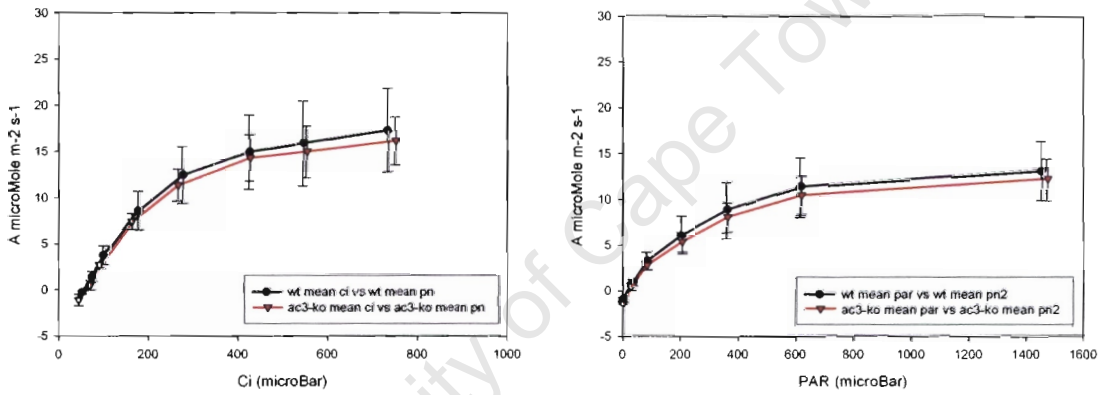
Surprisingly, at 5 weeks and under no dehydration stress, the *AC3* knock-out plants were photosynthesizing more efficiently than the WT plants (Fig. 7.8). In the CO₂ curve, at fully hydrated state, maximum assimilation (A_{\max}) for WT plants was $\sim 15 \mu\text{mol m}^{-2} \text{s}^{-1}$, while for *AC3* knock-out plants, A_{\max} was $\sim 20 \mu\text{mol m}^{-2} \text{s}^{-1}$. Similarly in the light curve, at full hydration, A_{\max} for WTs was $\sim 13 \mu\text{mol m}^{-2} \text{s}^{-1}$, while knock-out plants had an A_{\max} of $\sim 18 \mu\text{mol m}^{-2} \text{s}^{-1}$. At a semi-dehydrated state (31.3% SWC), this difference in A_{\max} for both curves in both plant types was significantly reduced. Both the WT and the knock-out plants photosynthesized at almost the same rate while in a semi-dehydrated state.

As dehydration proceeded the SWC lowered even more (14.8%). As a result the photosynthetic machineries of the plants became impaired and photosynthesis was very much reduced. In both the CO₂ and light curves, the knock-out plants' mean assimilation was less than the WT plants. Although they started off photosynthesizing very efficiently at full hydration, even better than WTs, the knock-out plants were photosynthesizing less efficiently than WTs at the end of the experiment when dehydrated.

Hydrated state: 52.9% SWC



Semi-dehydrated state: 31.3% SWC



Fully dehydrated state: 14.8% SWC

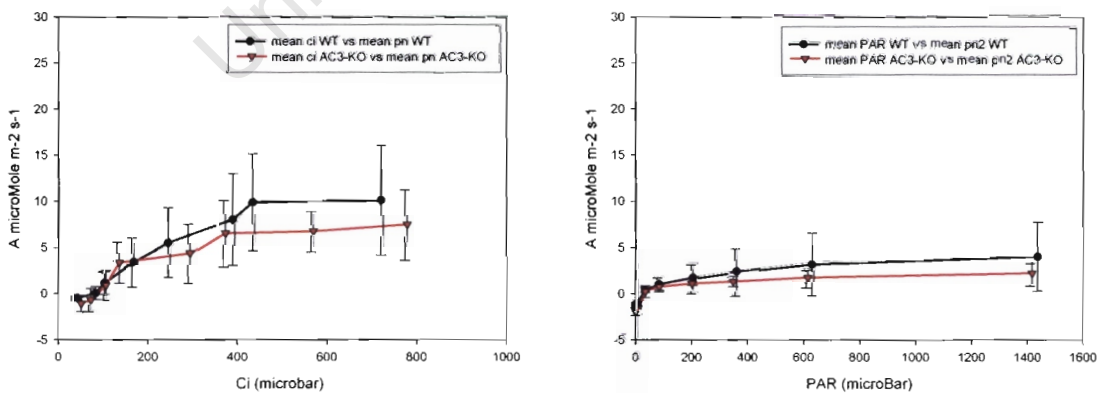


Figure 7.8. Mean Gas exchange activities of dehydrating WT Arabidopsis plants (●) compared to homozygous *AC3* knock-out plants (▼). Each data point is the mean of four individual plants.

7.3.6 Chlorophyll and Protein contents of WT and *AC3* Knock-out plants

To further investigate any physiological differences of *AC3* knock-out plants as compared to WT, the chlorophyll and protein contents from the two types of plants were measured and compared. The concentration of chlorophyll-*a* has been shown to relate to primary productivity (Wetzel, 1983) and can be used to assess physiological health by examining its degradation product, phaeophytin. Phaeophytin was extracted from leaf discs using 1 M perchloric acid and the pellet resuspended in 80% acetone. As shown in Figure 7.9, phaeophytin levels in *AC3* knock-out plants were slightly higher than those in WT plants, although the difference was very small.

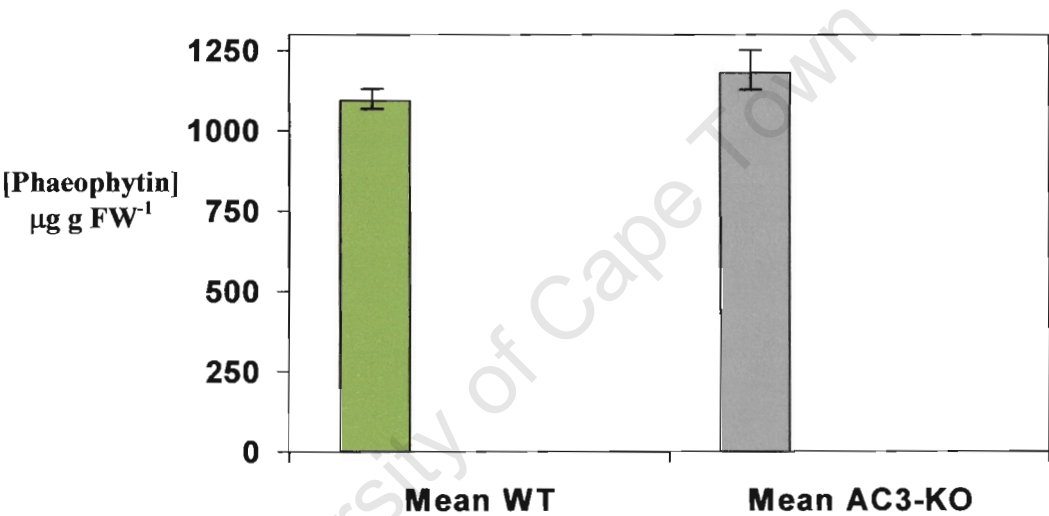


Figure 7.9. Phaeophytin levels in WT Arabidopsis plants (green bars) and homozygous *AC3* knock-out plants (grey bars). Error bars denote standard deviation of four replicates.

Similarly, the total protein content of WT and homozygous *AC3* knock-out plants were measured and compared. No significant differences could be observed between the two types of plants (Fig. 7.10). The total protein contents for both types were similar.

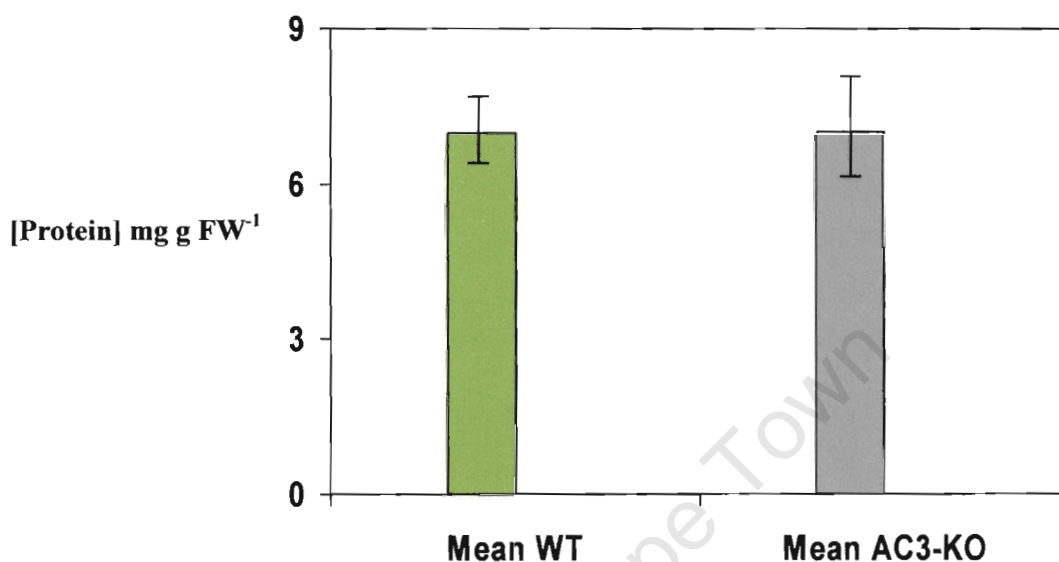


Figure 7.10. Total protein levels in WT (green bars) and homozygous *AC3* knock-out (grey bars) Arabidopsis plants. Error bars denoted standard deviation of four replicates.

7.3.7 Selecting and Testing for Arabidopsis *AC3* Over-expressor and *AC3* Antisense plants

A difference between putatively transformed and untransformed plants could be observed about 14 days after seed plating. While untransformed plants stopped growing and started to lose chlorophyll due to the kanamycin selection, putative transformed plants grew bigger and developed longer root systems (Fig. 7.11). Putative transformants were transferred into soil and grown in a controlled environment.

Testing by PCR

After the plants were three weeks old, genomic DNA was isolated and PCR performed to verify the insertion of the 35S-*AC3* construct in the genome. To verify *AC3* over-expressor (*AC3*-OE) plants, i.e., where the *AC3* cDNA was cloned in the sense orientation, a 35S-promoter specific forward primer was used and an *AC3* reverse primer. Figure 7.12 is a representation of the result obtained from testing a

few of the putative transformants. WT Col 0 DNA was used as a negative control to be sure that the amplification observed was specific to the site of insertion of the *AC3* cDNA into the genome. Actin forward and reverse primers were also used on the same genomic DNA samples in separate PCR reactions. This was to make sure that the template integrity was intact and fit for PCR amplification. Almost 100% of the plants tested by PCR were positive for the 35S-*AC3* insertion.

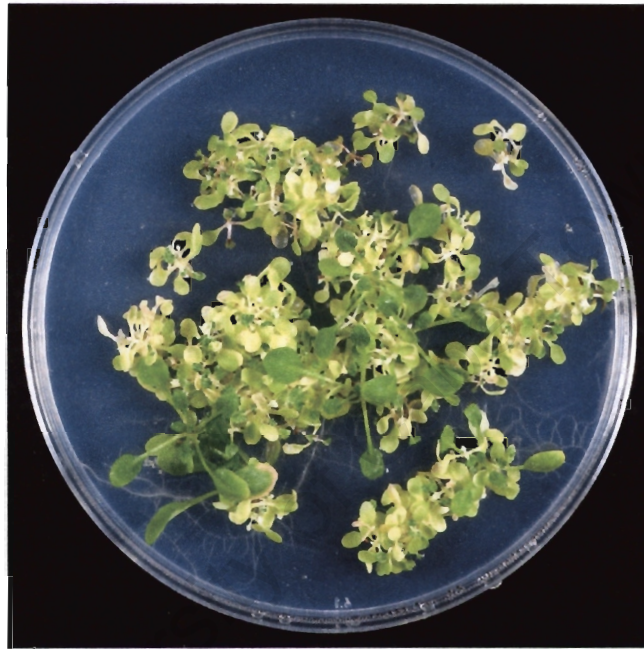


Figure 7.11. Selection for *Arabidopsis* transformants on full-strength MS plates containing kanamycin (50 $\mu\text{g/ml}$). Germination for 14 days: untransformed plants stopped growing and lost chlorophyll; putative transformed plants were bigger and greener with longer root systems.

Similarly, for PCR testing of *AC3* antisense transgenic plants (*AC3*-AS), genomic DNA was isolated and PCR performed using the 35S-promoter specific forward primer and the *AC3* specific primer with an integrated *Xba*I site previously used for cloning the *AC3* cDNA into pBin19-35S in the reverse orientation (section 7.2.3). The results are illustrated in Figure 7.13.

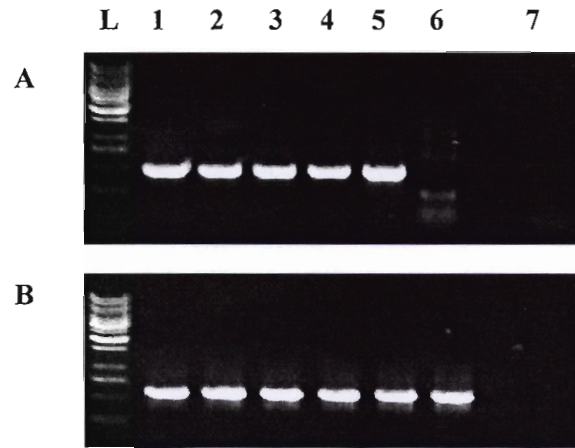


Figure 7.12. Visualization on 1.0% TAE EtBr-stained agarose gel of PCR on genomic DNA isolated from putative AC3-OE Arabidopsis transformants. L: 1 Kb ladder (Fermentas, USA). Lanes 1-5: Five independent putative AC3-OE lines (1-1, 2-3, 4-4, 5-3 and 6-2). Lane 6: WT Col 0 Arabidopsis. Lane 7: PCR negative control (no template). **A:** PCR using 35S-promoter forward primer and *AC3* cDNA specific reverse primer. **B:** PCR using actin forward and reverse primers.

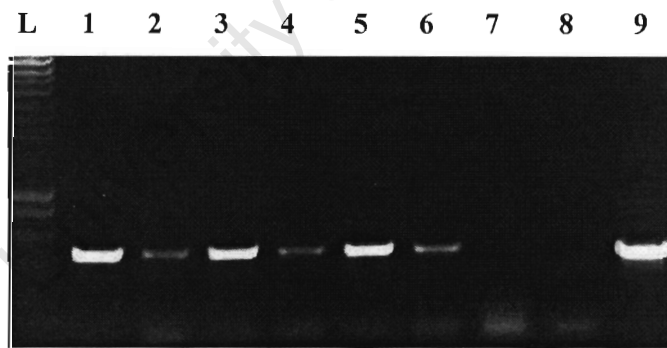


Figure 7.13. Visualization on 1.0% TAE EtBr-stained agarose gel of PCR on genomic DNA isolated from putative AC3-AS Arabidopsis transformants. L: HyperLadder I (Bioline, UK). Lanes 1-6: Five independent putative AC3-AS lines (1, 2, 3, 4, 5 and 6). Lane 7: WT Col 0 Arabidopsis. Lane 8: PCR negative control (no template). Lane 9: Positive control (pBin19-35S-AC3-AS template).

By northern blot analysis

After confirmation of the integration of the 35S-AC3 fragment into the plant genome had been established by PCR, the next step was to investigate firstly whether the transcript was being transcribed into mRNA and secondly, what the level of transcription was. Ten plants from the progenies of seven independent transformation events (T_1) were chosen for this purpose. Total RNA was isolated from hydrated leaf tissues of which 10 μ g each were separated by electrophoresis and transferred onto a nylon membrane. This membrane was probed with 32 P-dCTP labelled *AC3* cDNA. Transcription of the *AC3* mRNA transcript was observed and the level of expression varied from plant to plant (Fig. 7.14). No expression of *AC3* was detected in WT Col 0 Arabidopsis. Lines 3-2, 5-4, 5-6 and 7-4 were considered high over-expressors of *AC3*, lines 1-4, 3-5, 6-4 and 8-6 intermediate over-expressors and lines 2-4 and 2-5 low over-expressors. For future analyses, four *AC3* over-expressor lines were selected, representing all three categories of expression levels: lines 3-2, 5-4, 2-5 and 8-6.

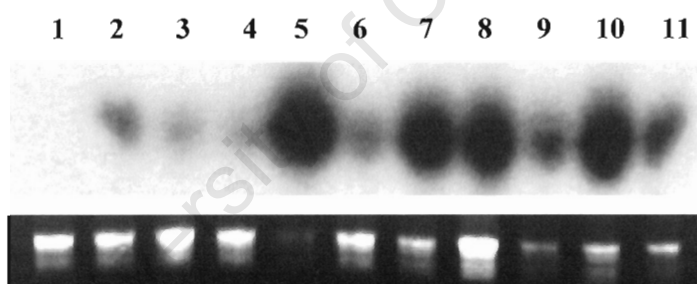


Figure 7.14. Northern blot analysis of *AC3* mRNA expression in transgenic Arabidopsis *AC3* over-expressors. Lane 1: WT Col 0 Arabidopsis. Lanes 2-11: Ten different (T_1) *AC3* over-expressor lines; 1-4, 2-4, 2-5, 3-2, 3-5, 5-4, 5-6, 6-4, 7-4 and 8-6 respectively. Lower panels represent ribosomal RNA bands on an EtBr-stained 1.2% agarose gel for comparison of RNA loading.

7.4 DISCUSSION

As mentioned previously, the precise function of LEAs remains unknown. Their involvement in stress tolerance is very likely since tomato, wheat and barley LEA proteins conferred increased resistance to osmotic or freezing stresses when yeast complementation studies were carried out (Zhang et al., 2000).

Recently a new member of a group 3 LEA, *cm80lea3*, was identified from the halophyte green algae *Chlamydomonas* sp. strain W80 (Tanaka et al., 2004). The cDNA was isolated based on the acquisition of NaCl salt-tolerance of the fresh water cyanobacterial cells carrying the gene. A local alignment between *cm80lea3* and AC3 using LALIGN (<http://au.expasy.org/tools/>) gave a 55.6% identity in a 9 amino acid overlap, 46.2% identity in a 13 amino acid overlap and 25% identity in a 20 amino acid overlap. However, AC3 and *cm80lea3* only shared ~30% identity overall.

Since AC3 was isolated because of its ability to detoxify H₂O₂ by complementation of an oxidative stress yeast mutant (*Δyap1*), the same mutant was used in further toxicity tests using the pro-oxidants menadione, diamide, tBOOH and as well as H₂O₂ to confirm the initial result. The expression of AC3 rescued the *Δyap1* phenotype when plated on media containing 1.1 mM diamide, 7.5 μM menadione and 55 μM tBOOH (Fig. 7.4). Phenotype rescue on media containing menadione was more pronounced while no rescue was observed on media containing H₂O₂. The activation of AC3 might therefore be specific to certain types of ROS only. A topology search of the AC3 amino acid sequence using PSORT prediction gave a score for both mitochondrial and chloroplast localization, with the higher score for localization in the chloroplast stroma (<http://psort.ims.u-tokyo.ac.jp/>). Therefore the action of AC3 might be restricted to the chloroplast.

Interestingly, AC3 knock-out Arabidopsis plants appeared to have slightly higher photosynthetic efficiencies than WT plants under control conditions (Fig. 7.8). However, while in a semi-dehydrated state (~30% SWC), the photosynthetic efficiency of both plants equalised. In a more advanced stage of dehydration (~15% SWC), photosynthesis collapsed in both types of plants, but was slightly more pronounced among AC3 knock-out plants. Taking the error bars into account, the

difference observed in the photosynthetic rate between WT and knock-out plants when fully hydrated do not seem to be significant. However, despite repetitions of the experiment using multiple replicates of each plant type, the difference observed remained consistent. If *Arabidopsis* indeed photosynthesize more efficiently under normal conditions in the absence of AC3, this implies a role for AC3 in the control of proteins involved in photosynthesis. *Arabidopsis* retains its chlorophyll during dehydration and the *AC3* steady-state mRNA transcript is up-regulated during drought stress (Fig. 6.7, section 6.3.4). Therefore as a protection mechanism, AC3 might be involved in suppressing photosynthesis during the stress. However, further physiological analyses did not produce any further significant differences between WT and *AC3* knock-out plants. Although the chlorophyll content of knock-out plants were higher than in WTs, the difference was too small to be statistically significant (Fig. 7.9). Similarly, no difference in total protein content was found (Fig. 7.10). It is important to note that Southern blot analysis revealed multiple insertions of the T-DNA elsewhere in the genome besides the *AC3* loci (Fig. 7.6). Although the pattern of growth of knock-out plants were similar to that of WT plants, one cannot confirm that any observations made in the knock-outs are genuine since T-DNA inserts elsewhere in the genome might be affecting the response of the plant (positional effect). Back-crossing of the knock-out with the WT will be carried out in order to eliminate undesirable T-DNA inserts until an *Arabidopsis* knock-out with a single insertion of T-DNA in the *AC3* loci is obtained. More physiological and molecular tests are required, on both knock-out and over-expressor plants, before a putative but more definite role can be assigned to AC3. However, thus far, from the yeast toxicity data, it is safe to speculate that AC3 is involved in protection against oxidative stress, more specifically against damage caused by superoxides. This is the first report of a LEA-like protein involved in such an activity.

Arabidopsis plants over-expressing and under-expressing (by antisense) the *AC3* gene have been generated to further investigate the function of this small stress-inducible LEA like protein. Due to time constraints, such experiments will not be included in this dissertation but will be carried out in the near future.

CHAPTER EIGHT

A COMPARATIVE ANALYSIS OF THE *X. VISCOSA* 1-CYS PEROXIREDOXIN WITH ITS ARABIDOPSIS ORTHOLOGUE

SUMMARY

The Arabidopsis *AtPer1* cDNA coding for a 1-Cys peroxiredoxin and its orthologue *XvPer1* from the resurrection plant *X. viscosa*, share 72% identity at the amino acid level. *AtPer1* was isolated by RT-PCR on cDNA generated from mRNA isolated from Arabidopsis seeds. Sequence analysis revealed that it is 648 bp long and codes for a protein of 216 amino acids with a molecular weight of 24 kDa. Two possible N-myristoylation sites, five possible casein kinase II phosphorylation sites and three possible protein kinase C phosphorylation sites were identified. A topology search suggests cytoplasmic location and a hydrophathy plot shows that *AtPer1* is hydrophilic. Both RT-PCR and northern blot analysis confirmed that *AtPer1* steady-state mRNA transcript was present only in Arabidopsis seeds under optimal growth conditions. Unlike *XvPer1*, *AtPer1* was not induced by dehydration stress. Transgenic Arabidopsis plants over-expressing the *X. viscosa* orthologue, as well as Arabidopsis plants under-expressing its own 1-Cys peroxiredoxin were developed. T₁ generation seeds for both transgenic types have been collected for further analysis.

8.1 INTRODUCTION

The 1-Cys peroxiredoxin from the resurrection plant *X. viscosa*, *XvPer1*, was found to be highly induced in vegetative tissues by heat, ABA, salinity as well as dehydration stresses (Mowla et al., 2002). Additionally, *XvPer1* was localized mainly to the nucleus of leaf tissues subjected to stress. It has been suggested that peroxiredoxins protect tissues from the damage caused by ROS (Haslekas et al., 2003a). The Arabidopsis 1-Cys peroxiredoxin, *AtPer1*, was first isolated by Haslekas et al. (1998). *XvPer1* and *AtPer1* share 70% identity at the amino acid level. *AtPer1* expression was found to be restricted to mature seeds and in situ experiments localized *AtPer1* to the vascular and epidermal tissues of the embryo and the aleurone layer. Transcript levels

of the gene remained high in an ABA-deficient *abal* mutant indicating that its expression was independent of ABA. The transcript could not be induced by either treatment with ABA or dehydration stress in the vegetative tissues. Over-expression of *AtPer1* in *E. coli* showed that the protein had anti-oxidant activity in vitro and protected the bacteria in vivo against the toxic effects of the pro-oxidant cumene hydroperoxide (Haslekas et al., 2003a).

Further studies on *AtPer1* expression and analysis of its promoter revealed that expression was controlled by ABI3, a transcriptional activator known to be required for ABA response in seeds (Haslekas et al., 2003b). Although ABI3 mediates the ABA response, it also regulates genes in an ABA-independent manner. The expression pattern of both genes coincided in seeds. The expression of a *GUS* gene fused to the *AtPer1* promoter showed that earlier statements of *AtPer1* being exclusively seed-specific were incorrect. Pronounced GUS expressions were seen in abscission zones of developing and mature siliques as well as at stem branching points of Arabidopsis WT seedlings. By doing northern blot analysis, very faint expression of *AtPer1* was detected in WT Arabidopsis seedlings treated with 3 and 10 μ M ABA as well as treatment with 0.1 to 1 mM of the oxidative stress inducer hydroquinone (HQ).

In Arabidopsis plants ectopically expressing *ABI3*, the *AtPer1* transcript accumulated to high levels when subjected to ABA, HQ and H₂O₂ stresses, which confirms the regulation of *AtPer1* by ABI3. Investigation of *AtPer1* promoter::*GUS* constructs in 35S::*ABI3* plants revealed that an intact ARE (Antioxidant Response Element) in the *AtPer1* promoter was necessary for ABI3-mediated oxidative stress induction of *AtPer1*.

Therefore, both *XvPer1* and *AtPer1* have been found to be stress-inducible genes, and particularly responsive to oxidative stress. However, the level of expression of *AtPer1* in vegetative tissues of WT plants is very low compared to *XvPer1*. Furthermore, the expression of *AtPer1* is dependent on the transcriptional activator, ABI3, which is only expressed in seeds. It is highly likely that *XvPer1* is also under the control of a similar transcriptional activator since, similar to the *AtPer1* promoter, the *XvPer1*

promoter also harbours an ARE element (Ingle et al., unpublished data). However, contrary to ABI3, the transcriptional activator of *XvPer1* is probably induced in vegetative tissues under stress conditions.

This study is mainly focused on the comparison of Arabidopsis plants over-expressing the *XvPer1* transcript in comparison with plants under-expressing *AtPer1*. The expression of *AtPer1* steady-state mRNA transcript in different parts of the Arabidopsis plant will be investigated using northern blot analysis and RNA from mature Arabidopsis plants. The expression of the transcript under dehydration stress will also be investigated. Transgenic Arabidopsis plants over-expressing the *XvPer1* transcript and *AtPer1* antisense plants will be generated for future analysis.

8.2 MATERIALS AND METHODS

8.2.1 Sequence Analysis of *AtPer1*

AtPer1 cDNA was isolated by RT-PCR using *AtPer1* specific primers (forward primer: 5'-GGTAAAATGCCAGGGATCAC-3'; reverse primer: 5'-TCAAGAGACCTCTGTGTGAC-3'). The primers were designed from the published *AtPer1* sequence (Haslekas et al., 1998; 0040005) and the transcript was isolated by PCR on cDNA generated from total mRNA isolated from dry Arabidopsis seeds. The nucleotide sequence of the *AtPer1* cDNA was determined on both forward and reverse strands using Dye Terminator sequencing (Oxford University, UK). The inferred amino acid sequence of *AtPer1* was obtained by translation of the cDNA sequence using the DNAMAN software (Version 3.0, 1997). The Bioinformatics and Biological Computing Unit, Weizmann Institute of Science, (Israel) was used to plot the hydrophilicity/hydrophobicity graph. The ScanProsite tool provided by ExpASY (us.expasy.org) was used to scan *AtPer1* for conserved motifs. A topology search was performed using iPSORT Prediction (www.psорт.org).

8.2.2 Plant Material and Growth Conditions

As outlined in section 6.2.2

8.2.3 Dehydration Stress Treatment

The dehydration treatments were performed in a controlled environment cabinet with growth chamber maintained at 22°C, a relative humidity of 50%, a day length of 16 hours and light intensity of 250 $\mu\text{molm}^{-2}\text{s}^{-1}$. Leaf samples were taken by cutting off whole leaves (3-5 leaves; mid-age), immediately wrapping in aluminium foil, flash-freezing in liquid nitrogen and subsequently storing at -80°C until nucleic acid isolation. SWC was measured using the following formula: $[(Fw-Dw)/ Fw] \times 100$. Fw is fresh weight and Dw is dried weight (in 80°C oven overnight). The dehydration stress was carried out on 5-weeks old plants by withholding water from the soil over a period of ± 15 days until the SWC was below 20% and the plants looked completely dehydrated.

8.2.4 Total RNA Isolation

As outlined in section 6.2.5

8.2.5 Northern Blot Analysis

As outlined in section 6.2.6

8.2.6 RT-PCR

As outlined in section 6.2.7

8.2.7 Development of *XvPer1* Over-expressor and *AtPer1* Under-expressors *Arabidopsis* plants

Cloning of XvPer1 and AtPer1 in pBin19-35S

Using *XvPer1* specific primers with incorporated enzymes sites (BamHI in the forward primer and XbaI in the reverse primer; primer sequences outlined in section 2.2.4) the *XvPer1* fragment was amplified by PCR using *pfu* DNA polymerase (Promega, UK) for high fidelity amplification. The amplified fragment was cloned

into pGem-T-easy and then into pBin19-35S. The strategy used was as outlined in section 7.2.7.

Primers specific to *AtPer1* were designed with integrated restriction sites which allowed for the cloning of the cDNA in the reverse orientation in pBIN19-35S. An *AtPer1* forward primer was designed with an XbaI restriction enzyme site and an *AtPer1* reverse primer was designed with a BamHI restriction enzyme site. The PCR-generated fragment was first cloned into pGem-T-easy and the cloning proceeded as described in section 7.2.7.

8.2.8 Testing for Positive Transformants

Preparation of electro-competent *Agrobacterium* bacterial cells, electroporation of *Agrobacterium* and transformation of *Arabidopsis* plants were as described in sections 7.2.8, 7.2.9, 7.2.10.

Putative transformants were tested by PCR on genomic DNA. DNA isolation was as outlined in section 7.2.3. For identifying *XvPer1* over-expressor plants, 35S-promoter specific forward primers and *XvPer1* specific reverse primers were used. For identifying *AtPer1* antisense plants, 35S-promoter specific forward primers and *AtPer1* specific reverse primers were used. The results were viewed on EtBr-stained agarose gels.

8.3 RESULTS

8.3.1 *AtPer1* Sequence Analysis

AtPer1 has an ORF of 648 bp, representing a deduced amino acid sequence encoding a protein of 216 amino acid residues with a molecular weight of 24 kDa and a predicted pI of 6.13 at pH 7.0 (Fig. 8.1). A scan tool suggests five possible casein kinase II phosphorylation sites at positions 5-8, 17-20, 71-74, 77-80 and 186-189 and three possible protein kinase C phosphorylation sites at positions 77-79, 143-145 and 205-207 (Fig. 8.1). A topology search revealed that it was most likely to be a cytoplasmic protein. A hydropathy plot showed that the protein was mostly hydrophilic (Fig. 8.2).

```

1  ATGCCAGGGATCACACTAGGAGACACGGTGCCGAACCTAGAAGTGGAGACGACACATGAC
1  M P G I T L G D T V P N L E V E T T H D

61  AAGTTCAAACCTTCATGACTACTTCGCCAATTCTTGGACCGTCCTCTTCTCATCCCGGG
21  K F K L H D Y F A N S W T V L F S H P G

121  GATTTACACCCGTGTGCACCACGGAGCTTGGTGCGATGGCCAAATACGCTCATGAGTTC
41  D F T P V C T T E L G A M A K Y A H E F

181  GATAAGAGAGGCGTGAAGCTCCTTGGTCTGTCTTGTGACGATGTACAGTCACACAAAGAC
61  D K R G V K L L G L S C D D V Q S H K D

241  TGGATCAAAGATATTGAAGCCTTTAATCACGGAAGCAAGGTGAATTACCCGATAATCGCT
81  W I K D I E A F N H G S K V N Y P I I A

301  GACCCGAACAAAGAGATCATTCTCAGCTTAACATGATTGATCCAATTGAGAACGGACCG
101  D P N K E I I P Q L N M I D P I E N G P

361  TCTCGTGCCCTTCATATTGTTGGTCTGACAGTAAGATAAAATTGAGCTTCTTGTATCCG
121  S R A L H I V G P D S K I K L S F L Y P

421  TCGACCACGGGACGTAACATGGACGAAGTATTGAGGGCTTTAGACTCGTTGTTGATGGCG
141  S T T G R N M D E V L R A L D S L L M A

481  TCCAAGCACAATAACAAGATCGCCACTCCGGTTAACTGGAAGCCAGATCAACCGGTTGTG
161  S K H N N K I A T P V N W K P D Q P V V

541  ATTTGCGCTGCTGTGTGCGACGAGGAAGCCAAAAGATGTTCCACAGGGTTTCAAGACC
181  I S P A V S D E E A K K M F P Q G F K T

601  GCCGATCTTCCGTCAAAGAAAGGCTATCTGCGTCACACAGAGGTCTCTTGA
201  A D L P S K K G Y L R H T E V S *

```

Figure 8.1. Coding nucleotide region and deduced amino acid sequence of *AtPer1*. The cDNA is 648 bp and codes for an amino acid sequence with an open reading frame of 216 amino acid residues and a molecular weight of 24 kDa. The casein kinase II phosphorylation sites are denoted by underlined residues. The protein kinase C phosphorylation sites are denoted as blue residues. * denotes the stop codon.

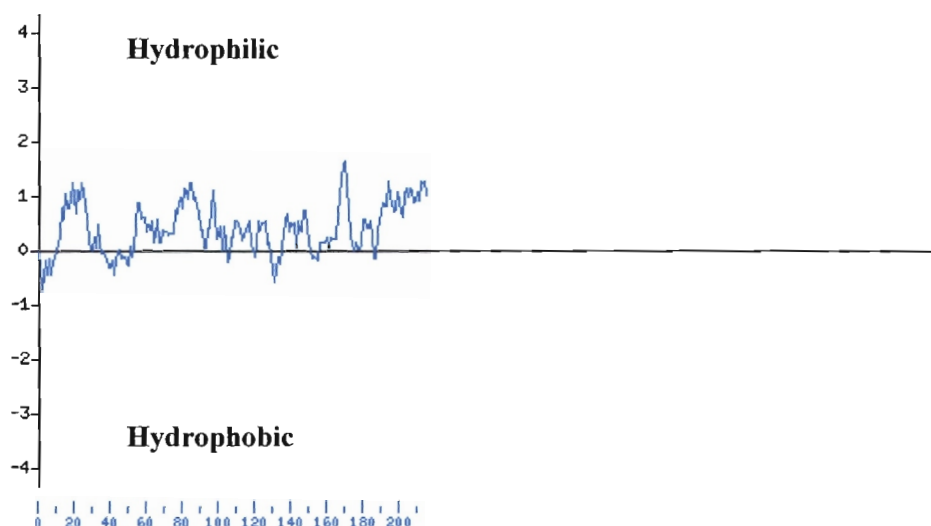


Figure 8.2. A hydropathy profile of the AtPer1 protein as determined by the method of Kyte and Doolittle (1982) using the Bioinformatics and Biological Computing Unit, Weizmann Institute of Science (Israel). The protein is mostly hydrophilic.

8.3.2 The Expression of AtPer1 in Different tissue types of Arabidopsis

Previous research has found that *AtPer1* is seed specific (Haslekas et al., 1998). Two methods were used here to investigate the expression of the *AtPer1* steady-state mRNA transcript in six different tissue types of the Arabidopsis plant under optimal conditions. RT-PCR was used first, using cDNA derived from total mRNA isolated from stem, leaves, flowers, immature siliques, dry mature seeds and imbibed seeds. *AtPer1* transcript was detected only in dry and imbibed seeds. No root tissue was available for this experiment.

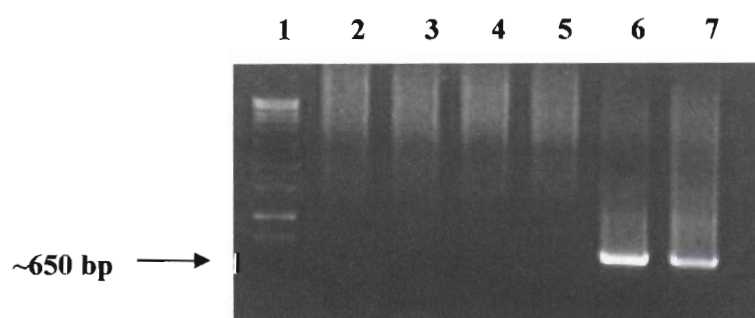


Figure 8.3. Visualization on 1.0% TAE EtBr-stained agarose gel of the expression of *AtPer1* mRNA in six tissue type of the Arabidopsis plant by RT-PCR. Lanes 1-6: 1 kb ladder (Fermentas, UK), stem, leaves, flowers, immature siliques, dry mature seeds and imbibed mature seeds respectively.

Northern blot analysis was also used for a more quantitative analysis. This time, root tissue was included. Again, *AtPer1* steady-state mRNA expression was only detected in dry seeds of Arabidopsis.

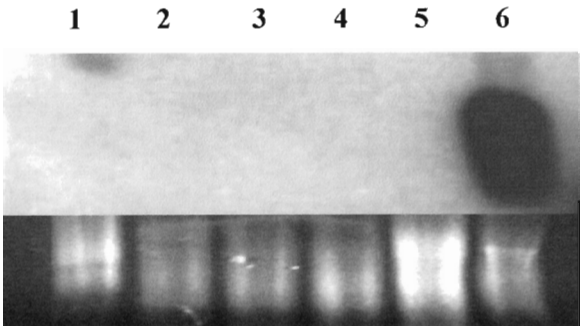


Figure 8.4. Northern blot analysis of *AtPer1* expression in different tissues of Arabidopsis. Lanes 1-6: roots, stem, leaves, flowers, immature siliques and dry seeds respectively. The lower panel represents ribosomal RNA bands on an EtBr-stained 1.2% agarose gel for comparison of RNA loading in gels.

8.3.3 Expression of *AtPer1* under Dehydration Stress

The expression of the *AtPer1* steady-state mRNA was investigated under dehydration stress since its *X. viscosa* orthologue was found to be highly induced by dehydration. The *AtPer1* transcript level was not induced by dehydration stress (Fig. 8.5).

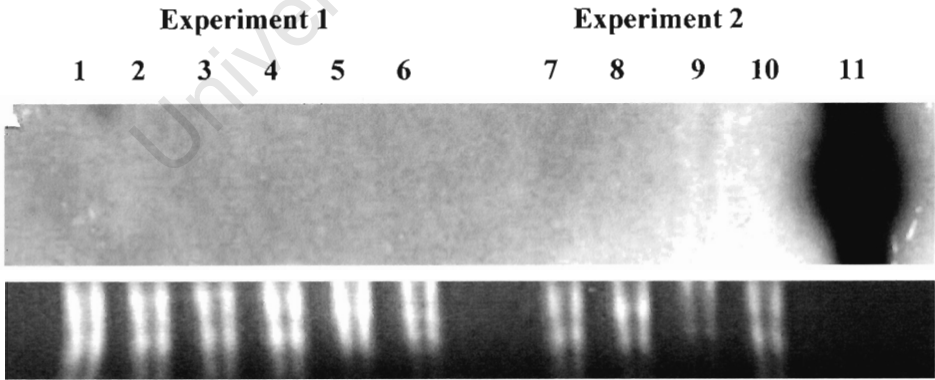


Figure 8.5. Northern blot analysis of *AtPer1* steady-state mRNA expression under dehydration stress in WT Arabidopsis (Col 0). Experiment 1; lanes 1-6: 65.0%, 53.0%, 46.0%, 33.4%, 22.8% and 12.0% SWC respectively. Experiment 2; lanes 7-10: 62.1%, 29.8%, 18.0% SWC and fully rehydrated respectively. Lane 11: Positive control (pGem-T-Easy-*AtPer1* plasmid). Lower panels represent ribosomal RNA bands on an EtBr-stained 1.2% agarose gel for comparison of RNA loading in gels.

8.3.4 Testing of putative *XvPer1* Over-Expressors using PCR

Putative transgenic plants were selected on media containing kanamycin selection and a difference between putatively transformed and untransformed plants could be observed ~14 days after seed plating. The putative transformants were transferred into soil and grown in a controlled environment. Genomic DNA was isolated from 3-week old plants and PCR performed using the 35S-promoter forward primer and *XvPer1* specific reverse primer (Fig. 8.6). All plants giving positive results were grown to maturity and seeds were collected.

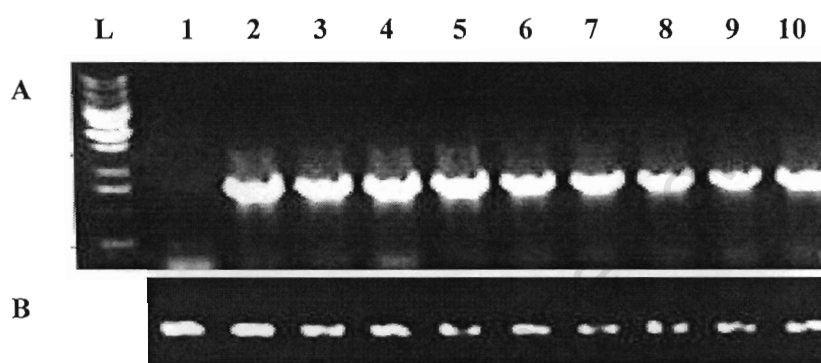


Figure 8.6. Visualization on 1.0% EtBr-stained agarose gel of the PCR on genomic DNA isolated from putative *XvPer1*-OE Arabidopsis transformants. L: 1 Kb ladder (Fermentas, USA). Lane 1: WT Col 0. Lanes 2-10: nine independent putative *XvPer1*-OE. A: PCR using 35S promoter forward primer and *XvPer1* cDNA specific reverse primer. B: PCR using actin forward and reverse primers.

Similarly, PCR was performed on genomic DNA isolated from putative *AtPer1* antisense plants using a 35S-promoter specific forward primer and an *AtPer1* specific reverse primer. All plants giving positive PCR results were grown to maturity and seeds were collected for further analysis.

8.4 DISCUSSION

The ability to be desiccation tolerant and dormancy are two very important and remarkable features of seeds (Aalen, 1999). Orthodox seeds can remain in a dry state of metabolic quiescence for long periods in contrast to the vegetative tissues of the majority of vascular plants. Resurrection plants, like seeds, have the ability to tolerate and survive desiccation (Scott, 2000).

One important aspect of desiccation tolerance is protection against the damaging effects of ROS accumulation (LePrince et al., 1994). Peroxiredoxins (Prx) are a novel family of thiol-dependent antioxidants which have been shown to be highly expressed in seeds and which can provide in vitro protection of enzymes, lipids and DNA against radical attack (Dietz, 2003; Kim et al., 1988; Lim et al., 1993; Netto et al., 1996).

The Arabidopsis 1-Cys Prx, *AtPer1*, was highly induced in seeds (Haslekas et al., 1998 and Fig. 8.4). However under conditions of dehydration stress, only very weak expression was observed in the vegetative tissue of seedlings and no expression in the vegetative tissues of mature plants (Haslekas et al., 2003b and Fig. 8.5). On the other hand, the 1-Cys peroxiredoxin from the resurrection plant *X. viscosa*, *XvPer1*, was found to be highly induced by dehydration stress (Mowla et al., 2002 and section 3.3.2, Fig. 3.5). Both proteins are very similar in size (~24 kDa). Both proteins have five possible Casein kinase II phosphorylation sites. Furthermore, both proteins are mostly hydrophilic. While *XvPer1* has been convincingly localized to the nucleus, some doubts still exist about the localization of *AtPer1* (Mowla et al., 2002; Haslekas et al., 1998, 2003a). However, there are certain marked differences in the hydropathy plots. Unlike *XvPer1*, *AtPer1* lacks any significant hydrophobic domains. This might be due to the difference in localization; in the seeds for *AtPer1* and in the vegetative cells for *XvPer1*.

An analysis of ~2 kb of the upstream sequences of both genes revealed common elements to both promoters, such as ARE, ABRE, DRE, MYB and MYC elements (Ingle et al., unpublished data). ABI3, a transcriptional activator required for ABA response in seeds, was found to be the inducer of *AtPer1* expression (Haslekas et al., 2003b). It is therefore safe to assume that *XvPer1* has an inducer very similar to ABI3 which is stress-inducible and expressed in vegetative tissues of *X. viscosa*.

Due to time constraints, the investigation of *AtPer1* expression in ABA-treated (100 μ M) and oxidatively stressed vegetative tissues of mature Arabidopsis plants could not be performed. This investigation will be carried out in this ongoing research as well as the analysis of Arabidopsis transgenic plants over-expressing *XvPer1*, the *X. viscosa* 1-Cys Prx and plants under-expressing *AtPer1*.

CHAPTER NINE

GENERAL CONCLUSIONS

In the early 1990s, the UN Food and Agricultural organization estimated that there were about 800 million undernourished people in the world (Food and Agriculture organization, 1992). A sizeable proportion of the world's poor are undernourished because they cannot grow enough food (Dyson, 1999). And in many situations, this poverty is caused because their crop yields are meager and their agricultural output is low. Additionally, the world population is growing continuously and population growth is the most important factor influencing increase in global food production.

According to Dyson (1999), the food prospects look bleakest for sub-Saharan Africa and it is estimated that their population will roughly double by 2025. New and improved technologies are needed in order to address these issues. Plant biotechnology, and more precisely plant genetic engineering, is a discipline which has evolved rapidly over the course of the last 15 years. In South Africa, controlled field trials with genetically modified organisms began in 1990 of Bt-cotton and the year 1997 saw the commercial release of the crop (AfricaBio, 2004). Today, over 80% of cotton planted in South Africa is GM. In 1998, GM maize was approved for commercial release and in 2000, GM soybeans.

The African continent is one of the driest and hence plants are often exposed to drought and heat stresses. In the last 5 years, the focus has been on the development of plants that can tolerate environmental stresses such as heat and drought. Africa, being a continent of many extreme habitats, is home to a large number of indigenous plants which are able to survive extreme conditions. One example is the resurrection plant *X. viscosa* which can remain in a dehydrated state for long periods of time until conditions become favorable (Sherwin and Farrant, 1995). Such plants are being used as potential sources of genes that could confer tolerance to abiotic stresses in crop plants such as maize, an African staple food. In the first study of this research project, the expression of XvPer1, a stress-inducible 1-Cys peroxiredoxin antioxidant enzyme from *X. viscosa*, was investigated.

9.1 XvPer1 is Hydrophilic and potentially Nuclear Localized

XvPer1 was isolated by differential screening of an *X. viscosa* cDNA library and was induced by dehydration stress. Analysis of the cDNA sequence indicated a mostly hydrophilic ~24 kDa protein with a putative bipartite nuclear localization signal.

9.2 XvPer1 has High Similarities to other Plant 1-Cys Peroxiredoxins

The deduced amino acid sequence of XvPer1 showed 77% with the *Bromus secalinus* orthologue, 77% similarity with the *Hordeum vulgare* orthologue, 74% similarity with the *Oryza sativa* orthologue, 72% similarity with the Arabidopsis orthologue and 72% similarity with the *Fagopyrum esculentum* orthologue. The protein coded for a novel 1-Cys peroxiredoxin antioxidant enzyme thought to be involved in the detoxification of ROS (Haslekas et al., 1998). All the sequences have an absolutely conserved peptide (PVCTTE) proposed to constitute the active site of the enzyme. All the orthologues, except for XvPer1, were seed specific. XvPer1 is the only reported angiosperm 1-Cys Prx to be highly expressed in vegetative tissues under dehydration stress. Southern blot analysis revealed that *XvPer1* is most likely to be a single copy gene.

9.3 XvPer1 is Multiple Stress Inducible in *X. viscosa*

Analysis of the *XvPer1* steady-state mRNA transcript expression by northern blot analysis showed that the gene was not only dehydration stress inducible, but was also induced by heat (42°C), high light intensity (1500 $\mu\text{mol m}^{-2}\text{s}^{-1}$), endogenous ABA application (100 μM) and by salinity (100 mM NaCl). Western blot analysis revealed that the XvPer1 protein expression correlated with the patterns of expression of *XvPer1* transcripts under the same stress conditions except for high light stress. It was therefore suggested that *XvPer1* plays an important role in response to water deficit stress.

9.4 XvPer1 is Localized to the Nucleus

Immunofluorescence localization using hydrated and dehydrated *X. viscosa* leaf tissue confirmed that XvPer1 was absent in hydrated tissue and present in dehydrated tissue. XvPer1 was found to be localized to the nucleus. A second chromogenic immunolocalization study using ABA-treated *X. viscosa* leaf tissue revealed that the

protein was mostly distributed inside the nucleus of ABA-treated leaf cells. Some accumulation of the protein was also detected in the cytoplasm and in the cell wall but XvPer1 was mostly nuclear-localized.

9.5 *XvPer1* protects against High Light Stress

XvPer1 was transformed into maize HiII calli by particle bombardment and lead to the constitutive expression of the gene in the crop plant. T₁ generation plants subjected to high light stress (1000 $\mu\text{mol m}^{-2}\text{s}^{-1}$) exhibited a growth advantage over control plants which had retarded growth, curled leaves and bleaching. XvPer1 is therefore thought to be involved in the protection of DNA and other cellular structures against the deleterious effects of ROS produced by high light stress.

This result demonstrates that the constitutive over-expression of a single stress-inducible gene from a monocotyledonous resurrection plant into a monocotyledonous crop plant can improve the plant's ability to survive in conditions of environmental stress. Since this result was preliminary, further analysis of the XvPer1 maize transgenics will be carried out to further characterize this novel stress-inducible antioxidant enzyme.

9.6 Future Prospects for *XvPer1*

Another stress-inducible gene from *X. viscosa*, *XVSAP1*, has demonstrated striking improvement of both tobacco and Arabidopsis plants subjected to abiotic stresses (Garwe et al., unpublished data). The upstream sequences of both the *XvPer1* and the *XVSAP1* genes have been isolated and characterized in an attempt to isolate stress inducible promoters. Since stress tolerance has been described to be a multigenic trait, future research will focus on the multiple-expression of stress-inducible genes from *X. viscosa* into crop plants under the expression of a stress-inducible promoter. The promoters of both *XvPer1* and *XVSAP1* have already been isolated and are being analysed.

9.7 AC3 is a Small Hydrophilic Protein belonging to group 3 LEAs

The second study of this research project involved the characterization of a small LEA-like protein from Arabidopsis. AC3 was isolated by its ability to confer H₂O₂

tolerance to baker's yeast. The cDNA codes for a small (~10 kDa) mostly hydrophilic protein containing a chloroplast signal peptide.

The deduced amino acid sequence of *AC3* shared 64% similarity with its tomato orthologue, 63% with the potato orthologue, 58% with the tobacco orthologue and 53% with both the *Ammopiptanthus mongolicus* and the mung bean orthologue. All of the orthologues are members of the group 3 LEA proteins. They are highly abundant during late embryo development (Wise and Tunnacliffe, 2004). However, their function remains obscure. They are thought to play a role in stress tolerance since many LEA proteins are induced by cold, osmotic stress and exogenous application of ABA (Welsh et al., 1994).

9.8 *AC3* is Not Seed-specific but is Dehydration and Oxidative stress Inducible

Northern blot analysis revealed that the *AC3* steady-state mRNA transcript was expressed mainly in roots, flowers and immature siliques of *Arabidopsis* under normal non-stress conditions. No expression was found in seeds.

Using northern blot analysis, *AC3* was found to be highly induced by dehydration stress in *Arabidopsis* leaf tissue. Exogenous application of ABA (100 μ M) also induced *AC3* but not dramatically. Oxidative stress imposed by the application of pro-oxidants (1 mM diamide, 10 mM H₂O₂, 50 μ M menadione and 100 μ M paraquat; independently) also induced *AC3* expression.

9.9 *AC3* Signal Transduction is ABA-independent

The *AC3* steady-state mRNA expression in both of the *Arabidopsis* mutants *aba1-1* (ABA-deficient) and *abi1-1* (ABA-insensitive) was highly induced under dehydration stress. This revealed that *AC3* expression was not dependent on the presence of ABA.

9.10 *AC3* relieves Oxidative Stress and may be Involved in Photosynthesis

Expression of *AC3* in the oxidative stress yeast mutant *$\Delta yap1$* improved its ability to survive on media containing the pro-oxidants diamide (1.1 mM), menadione (7.5 μ M) and ter-butylhydroperoxide (55 μ M). *AC3* knock-out *Arabidopsis* mutant plants photosynthesized more efficiently than WT plants under normal hydrated conditions.

However, under dehydration stress, both WT and knock-out plants photosynthesized at the same rate. No significant differences were found in the chlorophyll and total protein contents of WT and knock-out plants.

9.11 Future Prospects for *AC3*

Because of its distribution in the Arabidopsis plant under normal conditions, *AC3* is thought to be involved in the protection of roots, which senses dehydration stress first, and the reproductive organs, which are highly susceptible to even mild dehydration stress. Under conditions of dehydration and oxidative stresses, root-to-shoot signalling leads to the expression of *AC3* in vegetative tissues in an ABA-independent manner. *AC3* acts as an antioxidant involved in the detoxification of superoxides. *AC3* might also be involved in the regulation/protection of components of the photosynthetic machinery.

Southern blot analysis has revealed multiple T-DNA inserts in the *AC3* knock-out transgenic plant. Single T-DNA insert *AC3* knock-out plants will be selected by back-crossing. Transgenic Arabidopsis plants constitutively over-expressing *AC3* and *AC3* antisense plants have been generated for further analysis of the function of *AC3* and its involvement in oxidative stress tolerance.

9.12 A comparison of *AtPer1* and *XvPer1*

A comparison of the sequence of the two genes and their upstream sequences revealed many similarities despite the fact that one is seed-specific (*AtPer1*) and the other is highly induced by abiotic stresses in vegetative tissues (*XvPer1*). Transgenic Arabidopsis plants over-expressing *XvPer1* and Arabidopsis *AtPer1* antisense plants have been generated to further investigate these two genes.

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The End!

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